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Applicants:	C.A. Blau et al.	Attorney Docket No.:	UWOTL115624
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Title: METHODS OF CONTROLLING CELL DIFFERENTIATION AND GROWTH USING A FUSION PROTEIN AND A DRUG

INVENTOR'S DECLARATION UNDER 37 C.F.R. § 1.132

Seattle, Washington 98101

February 10, 2004

TO THE COMMISSIONER FOR PATENTS:

I, Dr. Carl Anthony Blau, declare as follows:

1. I am a co-inventor named in the above-identified patent application and I am familiar with the subject matter of this application.

2. My educational and work background is as follows: I received a Bachelor of Science degree from Wright State University in Dayton, Ohio, in 1982, and a Doctor of Medicine degree from Ohio State University in Columbus, Ohio, in 1986. I was an Intern in the Department of Medicine at Duke University in Durham, North Carolina, from 1986 to 1987, and a Resident in the Department of Medicine at Duke University in Durham, North Carolina, from 1987 to 1989. From 1989 to 1994, I was a Senior Fellow in the Division of Oncology at the University of Washington in Seattle, Washington. I am currently an Associate Professor in the Division of Hematology of the Department of Medicine and an Adjunct Associate Professor of Genome Sciences at the University of Washington, Seattle, and a Member of the Fred Hutchinson Cancer Research Center/University of Washington Cancer Consortium. I have extensive experience in gene transfer to and expansion of primary hematopoietic cells, such as hematopoietic stem cells.

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3. It is my understanding that U.S. Patent No. 5,741,899 (Capon et al.) has been cited as a prior art reference in the above-identified application.

4. The methods and cells claimed in this application, as amended, are directed to primary hematopoietic cells—specifically hematopoietic stem cells—containing a construct encoding a fusion protein comprising at least one signaling domain and at least one drug-binding domain, wherein exposure to a drug of primary cells transduced with this construct reversibly induces growth, proliferation, or differentiation of the cells. Capon et al. does not provide an enabling description that would permit one skilled in the art to make and use primary hematopoietic cells (such as primary hematopoietic stem cells) containing a construct encoding a fusion protein comprising at least one signaling domain and at least one drug-binding domain.

5. Capon et al. describes the construction of fusion proteins (chimeric proliferation receptors, CPRs) containing a drug-binding domain in EXAMPLE 7 (Col. 34, line 25, to Col. 36, line 12). Specifically, Capon et al. describes the construction of fusion proteins containing a $(FKBP)_3$ cassette consisting of 3 repeats of an FK506 binding protein module. All the fusion proteins explicitly described in Capon et al. that contain a domain capable of binding to a drug include this $(FKBP)_3$ cassette (see Col. 35, line 6-9, lines 18-21, lines 28-31, lines 38-41, lines 51-54, lines 61-64, and Col. 36, line 4-7).

6. Constructs that contain repetitive sequences, such as multiple copies of an FKPB domain, have a high frequency of recombination when introduced into cells using retroviral vectors, which very likely would compromise the function of the introduced sequences (see, e.g., Thomis et al. (2001) *Blood* 97(5):1249-1257, page 1251, Col. 1, Appendix 1). For this reason, the fusion proteins of Capon et al. including the $(FKBP)_3$ cassette would likely be rearranged by recombination and would likely be nonfunctional when introduced into cells using retroviral vectors.

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7. At the time of filing the application that issued as the Capon et al. patent (June 7, 1995), the only method for achieving stable gene delivery into primary hematopoietic cells was by using retroviral vectors (see, e.g., Brenner (1996) *N.E.J.M.* 335(5):337-9, page 337, Col. 2, paragraph 1, Appendix 2). Consistent with this, Capon et al. discloses the infection of human $CD8^+$ T cells with CPR-expressing retroviral vectors (Col. 41, lines 6-14). Because retroviral vectors were the only means of introducing constructs into primary hematopoietic cells in June 1995, and because repeated sequences have a high frequency of recombination in retroviruses, it is highly unlikely that the constructs containing 3 copies of the FKPB domain disclosed in Capon et al. would result in transduced primary hematopoietic cells expressing a functional fusion protein comprising a signaling domain and a drug-binding domain. For example, my group has found that a vector coding for a fusion protein containing three copies of the FKBP domain and the intracellular portion of c-kit can be used to achieve drug-induced proliferation when introduced into cell lines by electroporation (Jin et al. (1998) *Blood* 91(3):890-7, Appendix 3), whereas the same vector failed to provide drug-induced proliferation when introduced into the same cell lines by retroviral infection. Notably, Capon et al. does not describe expression in primary hematopoietic cells of a fusion protein containing a drug-binding domain, although it does describe the expression in $CD8^+$ T cells of other fusion proteins comprising cell surface receptors that bind the antigen CD4 (Col. 41, lines 44-60). For this reason, a person of skill in the art reading Capon et al. would not be able to practice without undue experimentation a method of rendering primary hematopoietic cells (such as primary hematopoietic stem cells) susceptible to drug-induced growth, proliferation or differentiation by expressing a functional fusion protein comprising at least one signaling domain and at least one drug-binding domain, or create without undue experimentation primary hematopoietic cells expressing a functional fusion protein comprising at least one signaling domain and at least one drug-binding domain.

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8. Moreover, Capon et al. does not provide an enabling description of methods of expanding primary hematopoietic cells (for example, primary hematopoietic stem cells) or methods of treating a hematopoietic disease or condition by exposing cells containing a construct coding for a fusion protein comprising at least one signaling domain and at least one drug-binding domain to the drug. Capon et al. describes placing CPR-expressing $CD8^+$ T cells in "culture dishes coated with saturating concentrations of either OCTK4A, anti-human Fc Mab, gp120, gp160-expressing cells, gp41/gp120-expressing cells, HIV-1 infected cells or FK1012" (Col. 42, lines 61-64). There are at least two reasons why this would not lead to drug-induced proliferation of the primary hematopoietic cells. First, coating the culture dishes with FK1012 would likely impede or prevent diffusion of the drug (FK1012) into the cells in order to bind to the drug-binding domains of the fusion proteins. Second, the use of "saturating concentrations" of FK1012 is problematic because too much FK1012 inhibits growth by occupying all of the FKBP sites, thereby preventing dimerization (see, e.g., Blau et al. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:3076-81, page 3078, Col. 1, of record). Thus, concentrations of FK1012 sufficient to saturate all of the FKBP sites would certainly not work. These issues, combined with the issue of recombination in the context of 3 FKBP domains in a retroviral vector, would clearly destine the method of obtaining drug-induced proliferation described in Capon et al. to failure. Therefore, a person of skill in the art reading Capon et al. would not be able to practice, without undue experimentation, methods of expanding primary hematopoietic cells—including hematopoietic stem cells—or methods of treating a hematopoietic disease or condition by exposing cells containing a construct coding for a fusion protein comprising at least one signaling domain and at least one drug-binding domain as described to a drug, and claimed in the above-identified patent application.

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9. Furthermore, the claimed invention provides methods for reversibly inducing proliferation of primary cells, and primary cells that can be reversibly induced to proliferate (see, e.g., Specification, page 25, lines 14-15; page 31, lines 23-34; page 42 lines 31-35). Capon et al. does not describe or suggest reversible induction of cell proliferation. To the contrary, the following passage illustrates that Capon et al. did not contemplate the reversible induction of cell proliferation:

> In addition to the gene encoding the chimeric proliferation receptor, additional genes may be included in the retroviral construct. These include genes such as the thymidine kinase or cytosine deaminase genes (Borrelli et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:7572) which acts as a suicide gene for the marked cells if the patient is exposed to gancyclovir or 5'-fluorouracil (5FU), respectively. Thus, if the percentage of marked cells is too high, gancyclovir or 5FU may be administered to reduce the percentage of cells expressing the chimeric receptors. In addition, if the percentage of marked cells needs to be increased, the multi-drug resistance gene can be included (Sorrentino et al. (1992) *Science* 257:99) which functions as a preferential survival gene for the marked cells in the patients if the patient is administered a dose of a chemotherapeutic agent such as taxol. Therefore, the percentage of marked cells in the patients can be titrated to obtain the maximum therapeutic benefit (Capon et al., Col. 22, lines 2-19).

This passage specifically demonstrates that Capon et al. did not contemplate that a small molecule inducer, such as FK1012, could be used to reversibly induce the growth of genetically modified primary cells. In contrast, the present application clearly points out the disadvantages to using cytotoxic drugs to selectively permit growth of only the cells transduced with a drug resistance gene:

> Conventional methods for selection involve the transfer of a gene encoding a product which confers resistance to a cytotoxic drug. Exposure of the cells to the corresponding cytotoxic drug permits selective growth of only those cells transduced with the drug resistance gene.
>
> The clinical applicability of this approach is limited by at least two factors. First, cytotoxic drugs have undesired consequences for the recipient when administered *in vivo*. Second, the persistent enrichment of genetically modified cells is expected to require that selection be exerted at the level of uncommitted cells. However, selective pressure is very difficult to apply at the level of progenitors and stem cell due to their intrinsic resistance to killing by most cytotoxic agents . . .

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New methods and materials for selecting a desired subpopulation of cells would be useful in a variety of biological research contexts and could be of particularly great value in clinical applications, especially if the use of cytotoxic drugs or other agents with unwanted pharmacologic activities can be avoided (Specification, page 1, line 20, to page 2, line 11).

Therefore, Capon et al. neither describes nor suggests reversibly inducing the growth of primary cells containing a construct coding for a fusion protein comprising at least one signaling domain and at least one drug-binding domain.

10. All statements made herein and of my own knowledge are true, and all statements made on information and belief are believed to be true; and further, these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issued thereon.

Respectfully submitted,

Dated: 2/10/04

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Dr. C.A. Blau

KBB:cj

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Appendix 1

A Fas-based suicide switch in human T cells for the treatment of graft-versus-host disease

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Graft-versus-host disease (GVHD) is a major complication of allogeneic bone marrow transplantation. One strategy to treat GVHD is to equip donor T cells with a conditional suicide mechanism that can be triggered when GVHD occurs. The herpes simplex virus thymidine kinase (HSV-*tk*)/ganciclovir system used clinically has several limitations, including immunogenicity and cell cycle dependence. An alternative switch based on chemically inducible apoptosis was designed and evaluated. A chimeric human protein was expressed comprising an extracellular marker (ΔLNGFR), the Fas intracellular domain, and 2 copies of an FK506-binding protein (FKBP). Primary human T lymphocytes retrovirally transduced with this construct could be purified to homogeneity using immunomagnetic beads. Genetic integrity of the construct was ensured by redesigning repetitive sequences. Transduced T cells behaved indistinguishably from untransduced cells, retaining the ability to mount a specific antiallogeneic immune response. However, they rapidly underwent apoptosis with the addition of subnanomolar concentrations of AP1903, a bivalent "dimerizer" drug that binds FKBP and induces Fas cross-linking. A single 2-hour treatment eliminated approximately 80% of T cells, and multiple exposures induced further apoptosis. T cells were eliminated regardless of their proliferation state, suggesting that the AP1903/Fas system, which contains only human components, is a promising alternative to HSV-*tk* for treating GVHD. (Blood. 2001; 97:1249-1257)

Introduction

In allogeneic bone marrow transplantation (BMT), the delayed infusion of donor lymphocytes plays a central therapeutic role in the control of disease relapse (graft-versus-leukemia effect [GVL])[1] and in the induction of immune reconstitution,[2,3] the latter especially in the subset of T-depleted matched transplants and in the context of partially mismatched transplants.[4] However, graft-versus-host-disease (GVHD) represents a frequent and often lethal complication of delayed lymphocyte infusions.[1] Managing the threat of GVHD while maximizing the beneficial GVL effect would broaden the scope and usefulness of allogeneic BMT procedures.

We and others have previously demonstrated that ex vivo genetic manipulation of donor lymphocytes to insert a conditional, drug-inducible suicide gene provides a means for the specific elimination of donor T cells with the onset of GVHD while maximizing the therapeutic benefit of the GVL effect.[2,5,6] Although a number of suicide genes have been proposed,[7,8] the herpes simplex virus thymidine kinase (HSV-*tk*)/ganciclovir (GCV)-based suicide strategy appears to be the most effective and specific and has been widely adopted.[9,10] Cells are engineered to express HSV-*tk*; the addition of ganciclovir leads to cell death through *tk*-catalyzed metabolism of the drug to a lethal product. In the current clinical trial,[6] HSV-*tk*-engineered–donor T cells demonstrated an effective antileukemic effect, and GVHD could be successfully treated through GCV administration.[2,11]

Despite this demonstration of efficacy, the study revealed limitations of the HSV-*tk*/GCV approach. First, in 8 of 24 treated patients, a specific cytotoxic CD8-mediated immune response developed against genetically engineered cells that led to the selective elimination of these cells (C.T., unpublished data). Although the cells expressed both HSV-*tk* and the cell surface marker ΔLNGFR (the extracellular and transmembrane domains of the human low-affinity nerve growth factor receptor), the immune response was directed exclusively against HSV-*tk*. This suggests that virally derived proteins were recognized by the immune system and eliminated, whereas ectopically expressed human products were not targets of immune recognition. Second, one patient with chronic GVHD exhibited a partial resistance to GCV-mediated elimination of transduced cells. This was attributed to the involvement of slowly proliferating lymphocytes in chronic GVHD and the cell cycle dependence of the HSV-*tk*–mediated killing. This suggests that the HSV-*tk* approach may have limited usefulness for the treatment of chronic GVHD.[11] Finally, some patients received GCV for concurrent clinical conditions other than GVHD, resulting in the expected, though undesired, clearance of HSV-*tk* donor lymphocytes.

To circumvent these limitations, we assessed the suitability of a novel suicide switch based on the human Fas receptor to trigger cell death in primary human T lymphocytes. Fas (CD95, APO-1) is a member of the tumor necrosis–nerve growth factor receptor superfamily.[12] Cross-linking of Fas results in the recruitment of a death-inducing signaling complex, activating a proteolytic cascade of caspases and inducing cell death by apoptosis.[12,13] We and others have previously described a system for activating apoptosis at will and demonstrated its function in engineered cell lines.[14,15] A

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chimeric protein containing the membrane-anchored intracellular domain of Fas is fused to the FK506-binding protein, FKBP12. Cross-linking of these proteins by the addition of a bivalent FKBP ligand (a "dimerizer") triggers the apoptotic death signal. Recently, we refined the system by designing a dimerizer drug, designated AP1903, with specificity for the engineered FKBP over the endogenous protein.[16]

In this report, we describe the experimental evaluation of the Fas-based suicide switch for the elimination of primary human T lymphocytes for the ultimate purpose of treating GVHD in BMT patients. At the beginning of this work we identified several features that would be required for successful use of the Fas switch—(1) efficient functional expression in primary human T lymphocytes; (2) very high purity of engineered cells to permit the quantitative elimination of cells in vivo; (3) genomic stability of the transgene, for the same reason; (4) rapid, efficient killing of engineered cells with low concentrations of drug; and (5) unaltered immune competence of engineered cells. We describe here the development and characterization of a Fas-based system that meets these criteria. We also use our experimental system to define a drug-dosing regimen that substantially eliminates all cells and, hence, is appropriate for clinical applications. The AP1903/Fas system is, therefore, a promising candidate for clinical use in controlling GVHD and should also be broadly useful for conditional ablation of T-cell subpopulations for research purposes.

Materials and methods

Retroviral plasmid construction

The construct LVVFas was derived from pSRα-LNGFR-2x(F36V-FKBP)-Fas-E[14,16,17] by removal of the C-terminal HA tag. LV′VFas was constructed by substitution of the first F36V-FKBP with the "codon-wobbled" F36V′-FKBP (see below). The construct LV′V, which lacks the cytoplasmic domain of Fas, was used as a negative control. The gene cassettes were ligated into the retroviral vector pMX[18] that was modified to include a puromycin resistance gene.

F36V′-FKBP construction

The codon-wobbled version of F36V-FKBP, called F36V′-FKBP, was constructed by a polymerase chain reaction (PCR) assembly procedure that contained 3 overlapping oligonucleotides and a 100-fold molar excess of 2 flanking primers (sequences available on request). The expected approximately 335-bp product was gel-purified, cloned, and sequenced to identify a correct clone.

Southern blot analysis

Genomic DNA was isolated from T lymphocytes by standard phenol–chloroform extraction. DNA was digested with *Xba*I, which cuts between ΔLNGFR and the first F36V-FKBP, and in the 3′ LTR. Blots (Hybond-N nylon membrane; Amersham, Buckinghamshire, United Kingdom) were hybridized with the *Xba*I–*Nae*I fragment of LV′VFas encoding for F36V′-F36V-Fas.

Polymerase chain reaction assay of FKBP region in Fas transgenes

Genomic DNA from Fas-engineered cells was prepared by digestion of 0.5×10^6 cells with 50 μg/mL proteinase K. Using a standard PCR reaction, the FKBP region of the Fas suicide cassette was amplified using 2 FKBP flanking primers (D. J. Talbot, unpublished data).

Retroviral transduction

Retroviral plasmids were introduced into amphotropic Phoenix cells (G. P. Nolan, Stanford, CA) by lipofection. Stable transfectants were identified by resistance to 50 μg/mL puromycin. Retroviral supernatants were used to infect human peripheral blood lymphocytes as follows. Peripheral blood was collected from healthy donors, and T lymphocytes were purified by Ficoll-Histopaque (Sigma, St Louis, MO) density centrifugation followed by magnetic selection using anti-CD3 Dynabeads (Dynal, Oslo, Norway). Bead-adherent cells ($2\text{-}4 \times 10^6$/mL) were cultured for 48 hours in RPMI 1640 supplemented with 10% fetal calf serum, 100 ng/mL anti-CD28 (Becton Dickinson, San Jose, CA), and 50 U/mL huIL-2. T lymphocytes (and NIH 3T3 cells) were transduced with retrovirus-containing supernatant either by plating on retronectin-coated wells (BioWhittaker, Walkersville, MD) or by centrifugation (90 minutes, 2000 rpm) in the presence of 4 μg/mL polybrene. Both methods gave similar transduction efficiencies; for T lymphocytes, this ranged from 10% to 50%.

Immunomagnetic selection of Fas-transduced T lymphocytes

Transduced T cells were immunoselected for expression of the cell surface marker ΔLNGFR as previously described.[2,6] Briefly, cells were incubated with mouse antihuman LNGFR monoclonal antibody 20.4 (Chromaprobe, Mountain View, CA) and selected with goat antimouse IgG-coated magnetic beads (Dynabeads M-450; Dynal). After overnight incubation at 37°C, magnetic beads were separated from the cells by pipetting and were removed magnetically from the culture. T lymphocytes purified in this manner were routinely more than 95% LNGFR-positive after a single round of selection. Cell recovery ranged between 40% and 50%.

Apoptosis–cell death assays

Elimination of T cells was measured by the following assay. Untransduced T cells were loaded with CellTracker Green CMFDA (Molecular Probes, Eugene, OR) as an internal control and were used to spike immunomagnetically sorted LV′VFas-transduced T cells. The cell mixture (2×10^6 cells/mL) was incubated with AP1903 for the indicated time, stained with 7-amino-actinomycin D (7-AAD, 2 μg/mL; Sigma) for 15 minutes on ice, and analyzed on a Becton Dickinson FACSort. The ratio (*R*) of live-gated (by forward/side scatter and 7-AAD), LV′VFas-transduced cells (nonfluorescent)–untransduced cells (green-fluorescent) was used to calculate specific cell survival using the following formula: %Survival = (*R*, drug-treated)/(*R*, untreated) × 100%. For annexin V assays, sorted LV′VFas-transduced T cells (2×10^6 cells/mL) were incubated with 10 nM AP1903. At the indicated time, an aliquot of 2×10^5 cells was taken, stained with annexin V–fluorescein isothiocyanate according to the manufacturer's instructions (Clontech, Palo Alto, CA), and analyzed by flow cytometry.

To compare the AP1903/FAS suicide system with the standard ganciclovir–HSV-*tk* system, human T lymphocytes transduced with the LV′VFas retroviral vector or with the SFCMM-3 retroviral vector[11] (carrying the HSV-*tk* gene) were immunoselected to more than 95% purity. Cells were cultivated for 5 days in the absence and in the presence of 10 nM AP1903 or 50 nM GCV in triplicate. [^{3}H]thymidine (1μ Ci/well; specific activity 87 Ci/mmol; Dupont, Boston, MA) was added 16 hours before harvesting the DNA and counting in a β-scintillation counter (Wallac, Turku, Finland, 1205 β-plate). The effects of the drugs on transduced lymphocytes were expressed as percentage of cell survival, referring to proliferation in the absence of the drug.

Analysis of antiallogeneic response

Antiallogeneic cytotoxic T lymphocytes were induced in vitro in a mixed lymphocyte reaction (MLR) with 2×10^6 LV′VFas-transduced donor effector lymphocytes or untransduced cells as control and 1×10^6 irradiated (6 Gy) allogeneic peripheral blood mononuclear cells (PBMC) (fully mismatched). The MLR was performed in Iscoves modified Dulbecco medium supplemented with 10% human serum, glutamine, and antibiotics in the presence of 50 U/mL huIL-2. Equal numbers of lymphocytes were tested in a standard cytotoxicity assay 10 days later, using as target cells ^{51}Cr-labeled phytohemagglutinin (PHA)–stimulated PBMCs from the same allogeneic donor and autologous PHA blasts as negative control cells. Natural killer cell–like activity was blocked by cold inhibition with a

30-fold excess of K562 cells over ^{51}Cr-labeled specific target cells. A secondary MLR was performed by the addition of irradiated allogeneic PBMC (1×10^6) in the presence or absence of 10 nM AP1903. Ten days later cells were counted, and the cytotoxic activity of equivalent numbers of cells was measured as above.

Results

Development of a conditional Fas suicide switch for use in primary human T cells

The original format of the inducible Fas suicide system was described by Spencer et al[15] and comprised the cytoplasmic domain of the human Fas death receptor fused to 2 copies of human wild-type FKBP12 and an epitope tag. This chimeric protein was fused to an N-terminal myristoylation motif to localize it to the plasma membrane. For this study, we made several modifications to optimize the construct for clinical use in T cells. We replaced the 2 FKBP12 domains with the FKBP point mutant, F36V-FKBP, to allow use of the more potent and specific dimerizer drug, AP1903.[16] We removed the C-terminal epitope tag to minimize the potential immunogenicity of the protein. In addition, we replaced the myristoylation motif with the extracellular and transmembrane domains of LNGFR to localize the chimeric protein to the plasma membrane and simultaneously provide a cell surface marker.[6,17] The approach physically links the conditional apoptosis cassette with an affinity handle that can be used to purify transduced cells, providing a means to ensure that all resultant cells can be eliminated by adding AP1903. The resultant gene cassette is herein called LVVFas (Figure 1A).

One of the major safety considerations of using any suicide gene approach for the treatment of GVHD is that all the ex vivo–engineered T lymphocytes to be infused into the patient must express the functional suicide gene. This is of particular concern with the AP1903/Fas system because of the presence of repetitive sequences (2 copies of F36V-FKBP).[16] Retrovirally introduced transgenes containing repetitive sequences tend to have a high frequency of rearrangement, which very likely would compromise the function of the chimeric Fas protein. Selection of transduced cells using the LNGFR surface marker does not necessarily prevent exclusion of cells expressing a rearranged transgene because the rearrangement may not affect the LNGFR portion of the protein. Previous studies that used the conditional Fas suicide approach did not report problems with rearrangement, but in these cases nonretroviral approaches or clones of transduced cells were used.[14-17,19]

To reduce the risk for rearrangement associated with the 2 identical copies of F36V-FKBP, we designed a "codon-wobbled" variant of F36V-FKBP, termed F36V′-FKBP. These 2 constructs encode identical polypeptide chains but share only 62% homology at the nucleotide level by virtue of silent changes of the wobble bases in most codons. The construct LV′VFas incorporates one copy of F36V′-FKBP and one copy of F36V-FKBP and is anticipated to be less recombinogenic than LVVFas (Figure 1A). A control construct, LV′V, lacks the cytoplasmic domain of Fas and thus is incapable of signaling. These transgenes were cloned into the retroviral vector pMX,[18] which uses the MoMLV LTR to drive expression and which was found to provide a higher-level expression of the chimeric protein than LXSN-based vectors (data not shown).

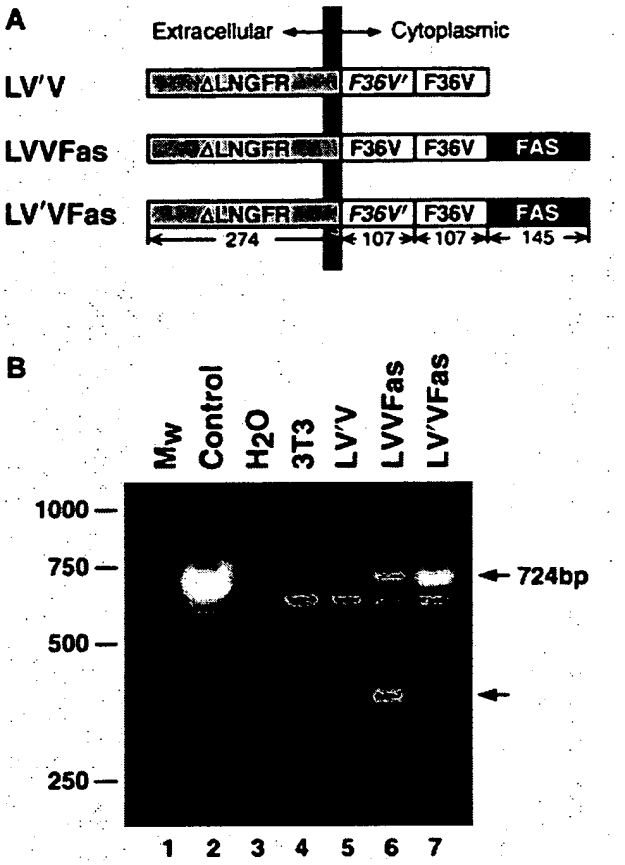

Figure 1. Fas constructs and PCR analysis. (A) Schematic of Fas constructs. The constructs LV′V, LVVFas and LV′VFas contain combinations of the following elements, as indicated: ΔLNGFR, the extracellular and transmembrane domains (residues 1-274) of the human low-affinity nerve growth factor receptor; F36V, Phe36 → Val36 point mutant of human FKBP12; *F36V′*, codon-wobbled F36V; FAS, cytoplasmic domain (residues 175-319) of human Fas. Sizes (in amino acids) of the individual components are indicated. Expression of the transgenes was driven by the MoMLV LTR using the retroviral vector pMX. (B) PCR analysis of FKBP region in Fas-transduced NIH 3T3 cells. Fas-transduced 3T3 cells were lysed, and the integrity of the FKBP region of the Fas transgene was analyzed by PCR. Lane 1, molecular weight standard; lane 2, control LV′VFas plasmid; lane 3, no DNA control; lane 4, untransduced 3T3 cells; lane 5, LV′V-transduced 3T3 cells; lane 6, LVVFas-transduced 3T3 cells; lane 7, LV′VFas-transduced 3T3 cells. Arrows on the right indicate the sizes of the expected band (724 bp) and a band truncated by approximately 300 bp.

F36V′-FKBP prevents rearrangement of the Fas transgene during retroviral transduction

To test the performance and genetic stability of these constructs, they were transiently transfected into amphotropic Phoenix packaging cells, and supernatant collected 48 hours later was used to transduce NIH 3T3 cells. To examine the integrity of the FKBP portion of the Fas transgene, we used a PCR-based assay in which the FKBP portion is amplified from the genomic DNA of transduced cells. Cells transduced with LV′VFas yielded a single PCR product of the expected size, 724 bp (Figure 1B, lane 7). However, cells transduced with LVVFas produced an additional band reduced in size by approximately 300 bp, a size difference consistent with the deletion of one FKBP (Figure 1B, lane 6). This result indicates that there is a significant frequency of rearrangement with the Fas construct containing 2 identical copies of F36V-FKBP but that the use of the modified F36V′-FKBP/ F36V-FKBP combination eliminates this problem.

Engineering of primary human T lymphocytes with Fas suicide genes and immunomagnetic selection of transduced cells

We next evaluated the expression and stability of the conditional Fas constructs in T cells. Primary human T lymphocytes were isolated from peripheral blood obtained from volunteer donors using anti-CD3 magnetic beads. Purified T lymphocytes were stimulated for 48 hours before infection with Fas recombinant retroviruses. We observed high-level expression of the transgene as monitored by flow cytometry to detect LNGFR-positive cells (Figure 2A), but there was a complete absence of "toxicity" of the construct (data not shown). To test whether the ΔLNGFR cell surface marker could be used to isolate transduced T cells, LNGFR-positive cells were immunoselected 48 hours after infection with anti-LNGFR magnetic beads as previously described.[2] We found that cells sorted in this manner were routinely more than 95% to 99% LNGFR-positive in a single round of purification (Figure 2A). Of note, cell viability was not compromised during the selection process (data not shown). This is an important observation because cross-linking of LNGFR by the antibody-coated beads might be expected to cluster the Fas domains and activate signaling, an outcome that would complicate the use of this procedure to isolate transduced cells. Similarly, ligation of LNGFR on the surface of LV′VFas-transduced T cells with human β nerve growth factor (β-NGF) had no effect on cell viability (data not shown). The fact that these procedures do not trigger apoptosis suggests that the cross-linking of LNGFR brings together the Fas death domains in a structural context that is unproductive for signaling.

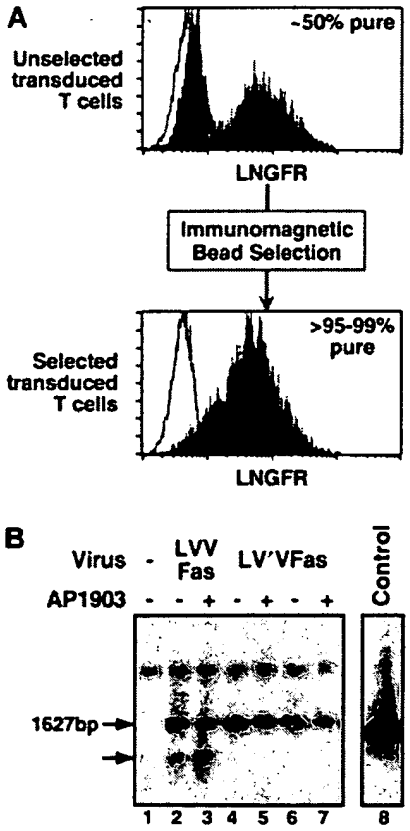

Figure 2. Immunomagnetic selection and Southern blot analysis. (A) Immunomagnetic selection of Fas-engineered T lymphocytes. Fas-transduced primary human T lymphocytes were selected with anti-LNGFR–coated magnetic beads, as described in "Materials and methods." Before selection, cells were 10% to 50% LNGFR-positive; after selection, they were routinely more than 95% to 99% pure. Histogram plots show the LNGFR staining profile of transduced (filled histograms) and untransduced cells (open histograms). (B) Southern blot analysis of Fas-transduced T lymphocytes. Immunomagnetically sorted, Fas-transduced primary human T lymphocytes were either challenged with 10 nM AP1903 for 10 days (+) or left unchallenged (−). DNA was digested with *XbaI*, separated by electrophoresis and probed with an FKBP–Fas-specific probe. Lane 1, untransduced control lymphocytes; lanes 2 and 3, LVVFas-transduced lymphocytes; lanes 4 and 5, donor A LV′VFas-transduced lymphocytes; lanes 6 and 7, donor B LV′VFas-transduced lymphocytes; lane 8, control plasmid. Arrows indicate the size of the expected band (1627 bp) and a band truncated by approximately 300 bp.

To assess the integrity of the Fas transgene in transduced human T lymphocytes, Southern blot analysis was performed. Similar to our findings in 3T3 cells, primary human T lymphocytes transduced with LVVFas yielded—in addition to a band of the expected size (1627 bp)—a band truncated by approximately 300 bp (Figure 2B, lane 2), probably representing the deletion of one FKBP. Challenge of LVVFas-transduced cells with AP1903 resulted in the enrichment of this fragment (Figure 2B, lane 3). This result suggests that approximately 10% to 15% of LVVFas-transduced T lymphocytes harbor a rearranged transgene and that these cells have lost sensitivity to AP1903-induced cell death. In contrast, cells transduced with LV′VFas showed no aberrant-sized bands in the absence or in the presence of AP1903 (Figure 2B, lanes 4-7), demonstrating the lack of rearrangement and confirming the integrity of the LV′VFas transgene. Based on these results, subsequent work was carried out exclusively with the LV′VFas construct.

LV′VFas transgene confers susceptibility to AP1903-induced cell death to primary human T lymphocytes

We next assessed the susceptibility of LV′VFas-transduced T cells to AP1903-induced cell death. The Fas system has previously been tested in vitro in several cell lines, including HT1080, 293, Jurkat, and HeLa cells,[14,16,17,19] and in vivo in a transgenic mouse model.[15] Interestingly, the killing efficiency varied widely in these diverse cell types, perhaps reflecting an intrinsic, cell type–specific susceptibility to Fas-induced apoptosis. This emphasizes that the performance of the Fas system must be optimized for each target cell type—in the case of this study, primary human T cells.

LV′VFas-transduced T lymphocytes were exposed to different concentrations of the dimerizer AP1903, and survival was analyzed 24 and 48 hours later. As shown in Figure 3A, the induction of cell death was highly dose dependent. Maximal killing occurred in the presence of 3 to 10 nM AP1903, and the IC_{50} was approximately 0.2 nM. In a large number of experiments, the maximal killing efficiency was consistently in the range of 60% to 80%, and the IC_{50} was reproducibly approximately 0.2 nM. We observed no significant donor-to-donor variability of the killing efficiency (data not shown). In addition, when CD4 and CD8 T-cell populations were examined separately, no difference was found in the killing efficiency or in the IC_{50} value (data not shown). This information is relevant because CD4 and CD8 donor lymphocytes contribute to GVHD, as shown by donor lymphocyte infusion protocols involving the infusion of either CD4-depleted[20] or CD8-depleted donor lymphocytes.[21] LV′VFas-transduced T lymphocytes demonstrated normal viability in the absence of drug, indicating the absence of autotoxicity—signaling in the absence of added drug (data not shown). AP1903 treatment of LV′V-transduced (Figure 3A) or untransduced control T lymphocytes (data not shown) had no significant effect on their survival, even at the highest concentration of AP1903 tested (1 μM, data not shown). These results demonstrate that the AP1903/Fas suicide system is highly effective and specific to T lymphocytes containing the Fas transgene.

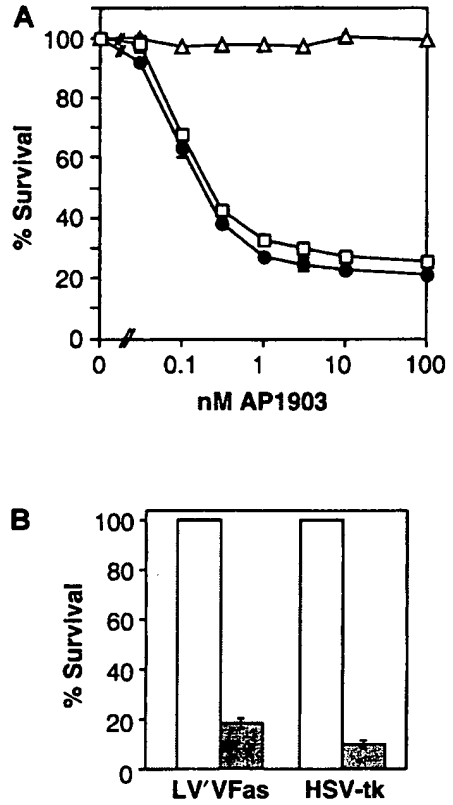

Figure 3. Killing of human T cells. (A) Dose-response curve of AP1903-induced killing of Fas-transduced human T cells. Primary human T lymphocytes were retrovirally infected, and transduced cells were immunomagnetically sorted. Cells were incubated with the indicated concentrations of the synthetic dimerizer AP1903, and specific survival was measured as described in "Materials and methods." Cells analyzed are control LV'V-transduced cells (△), LV'VFas-transduced cells analyzed after 24 hours (□), and LV'VFas-transduced cells analyzed after 48 hours (●). Values are the mean ± SD of duplicate points (except for LV'V). The result shown is representative of at least 5 independent experiments. (B) Comparison of killing of human T cells engineered with LV'VFas or HSV-*tk*. Primary human T lymphocytes were transduced with LV'VFas or SFCMM-3 (HSV-*tk*), immunomagnetically purified and treated with either 10 nM AP1903 or 50 nM GCV for 5 days. Survival was measured as described in "Materials and methods." Open bars represent untreated cells; filled bars represent cells treated with drug. Values are the mean ± SD of 3 independent points.

As shown in Figure 3B, the extent of cell death of drug-treated Fas-engineered T cells is comparable to that of HSV-*tk*–engineered cells. In this experiment a second-generation vector expressing wild-type HSV-*tk* (SFCMM-3) was used.[11] Because a previous version of this vector (SFCMM-2, which expresses HSV-*tk*-neo fusion) confers significantly lower sensitivity to GCV[11] but was used successfully to ablate T cells and treat GVHD in clinical trials,[2] these data suggest that the AP1903-Fas system may also be suitable for clinical use to treat GVHD.

Induction of apoptosis by the AP1903/Fas system occurs within 1 hour of drug addition

To provide rapid mitigation of GVHD, it is important that the donor T cells be neutralized as quickly as possible. In contrast to HSV-*tk*–induced cell death, Fas-induced apoptosis is expected to be a rapid event that occurs within hours of the appropriate signal. Some of the earliest detectable events in the apoptotic pathway are the loss of the cytoplasmic polarization of plasma membrane phosphatidylserine and the fragmentation of nuclear DNA.[13,22,23] We used the former readout to determine the kinetics of AP1903-induced cell death. LV'VFas-transduced T lymphocytes were treated with 10 nM AP1903 and stained with annexin V[24] at different times (Figure 4). In as little as 1 hour, apoptotic cells were clearly detectable. The percentage of apoptotic cells continued to increase over time; after 24 hours of drug treatment, 68% of the cells were apoptotic (Figure 4). In contrast, LV'VFas-transduced lymphocytes cultured in the absence of AP1903 showed only a small increase in the proportion of apoptotic cells that could be attributed to spontaneous cell death during culture (Figure 4). Similarly, no significant change in the annexin V staining profile was observed when untransduced T cells were incubated with AP1903 (data not shown). These findings show that AP1903-induced apoptosis is extremely fast. Furthermore, the kinetics observed with AP1903-induced apoptosis are similar to those of primary human lymphocytes (our unpublished observations) or the human Jurkat T-cell line treated with anti-Fas antibodies,[24] suggesting that the same signaling cascade is triggered by both agents.

Maximal killing is achieved even by short exposures to AP1903, and additional doses increase the efficiency

To explore the AP1903 dosing regimen and administration schedule likely to be required in a clinical setting, we determined the length of time Fas-engineered T cells must be exposed to the drug to trigger apoptosis. LV'VFas-transduced T lymphocytes were treated with 10 nM AP1903 for 1, 2, or 4 hours, the drug was washed away, and the cells were further incubated until analysis at 24 or 48 hours. Surprisingly, even a 1-hour exposure resulted in near-maximal cell death (Figure 5A). Prolonging the duration to 2 or 4 hours did not significantly increase the killing efficiency, and the continuous presence of the drug had only a small additional effect at the 48-hour time point (Figure 5A).

The fact that a single AP1903 dose eliminates 60% to 80% of engineered cells raises the question of whether the remaining cells are intrinsically resistant to AP1903-induced apoptosis. This is a

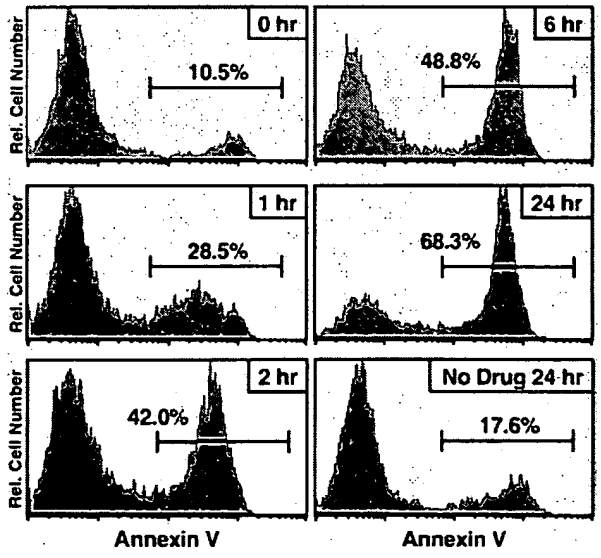

Figure 4. Annexin V kinetics of AP1903/Fas-induced apoptosis. LV'VFas-transduced primary human T lymphocytes were incubated with 10 nM AP1903, and aliquots of cells were stained with annexin V after 1, 2, 6, and 24 hours. As a control, cells were stained at the beginning of the treatment (0 hour) and after 24 hours in the absence of drug. Cells were analyzed by the use of flow cytometry, and the annexin V staining profile is shown. Percentages of annexin-V–positive (apoptotic) cells are indicated. Untransduced T cells incubated with 10 nM AP1903 showed no significant change in their annexin V staining profile (data not shown).

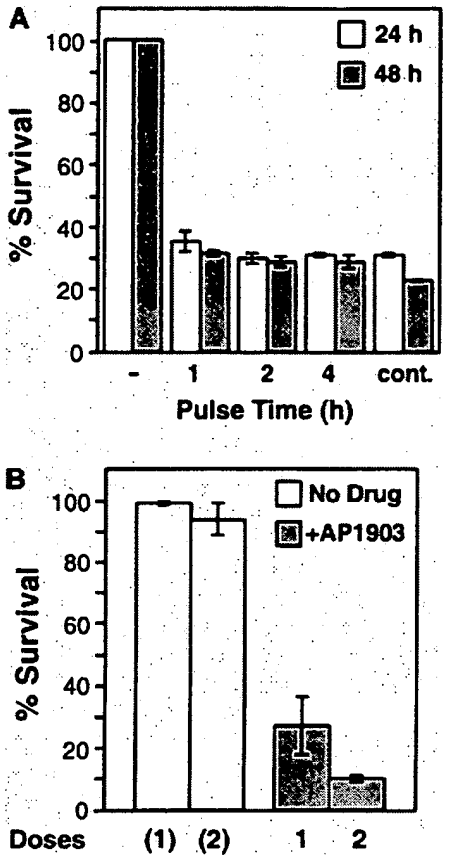

Figure 5. Short-term and multidose treatment. (A) Short-term treatment ("pulsing") of Fas-transduced T cells with AP1903. LV'VFas-transduced primary human T lymphocytes were incubated with 10 nM AP1903 for 1, 2, or 4 hours and were washed extensively, and survival was assayed after 24 (open bars) or 48 (shaded bars) hours, as described in "Materials and methods." As a control, cells were incubated in the absence of AP1903 (−) or in the continuous presence of 10 nM AP1903 (cont.). Values are the mean ± SD of duplicate points. (B) Multidose treatment of Fas-transduced T cells. LV'VFas-transduced T lymphocytes were treated once or twice (48 hours apart) without drug (open bars) or with two 2-hour pulses (doses) of 10 nM AP1903 (shaded bars). In parallel, as a control, cells were treated identically without the addition of drug (open bars). Survival was assayed 48 hours after drug treatment. Values are the mean ± SD of 2 independent experiments.

significant question because clinical use to mitigate GVHD would be optimal if all engineered cells could be eliminated. We investigated whether cells surviving the first drug challenge could be eliminated by a second drug administration. As shown in Figure 5B, a first dose of AP1903 (2-hour pulse with 10 nM) resulted in the elimination of approximately 72% of LV'VFas-transduced T lymphocytes. Forty-eight hours after the first dose, the surviving cells were treated with a second 2-hour pulse of AP1903. This second dose resulted in the killing of more than 60% of the remaining cells. Together, the 2 pulses (doses) resulted in an aggregate killing efficiency of approximately 90% (Figure 5B). In contrast, control LV'VFas-engineered cells subjected to the same procedure but without the addition of AP1903 showed only a small decrease in viability (Figure 5B). These findings indicate that cells not killed by the first AP1903 treatment have not acquired resistance and are only temporarily refractory to AP1903/Fas-induced cell death. This is supported by the absence of rearranged transgene-bearing cells before or after AP1903 treatment (Figure 2B, lanes 4 to 7). Rather, the presence of cells surviving AP1903 treatment is probably attributable to properties intrinsic to T-cell biology[25] and is consistent with previous observations that cross-linking of endogenous Fas on the surface of stimulated primary human T lymphocytes[26,27] or human Jurkat T cells[28,29] kills only 40% to 80% of the cells. Although the phenomenon is not yet fully understood, temporary resistance of T cells to Fas-induced cell death may reflect stochastic variations in intracellular levels of antiapoptotic molecules such as bcl-2 and bcl-x_L,[30,31] XIAP,[32] or c-FLIP,[33] leading to transient blockage of the Fas signal.

Interestingly, AP1903 treatment of Fas-engineered lymphocytes caused the rapid down-regulation of the LV'VFas protein in cells that were not killed by the drug (data not shown). This disappearance from the surface is dependent on the Fas signaling domain; the expression level of the LV'V control transgene is not affected by the drug (data not shown). However, the down-regulation of LV'VFas expression is only temporary. Expression levels recover within 24 to 48 hours after withdrawal of the drug (data not shown).

AP1903/Fas-induced cell death of primary human T lymphocytes is not dependent on the proliferation state of the cell

T lymphocytes are a highly heterogeneous population of cells with different antigen specificities, and they exist in various states of activation and differentiation. One of the inherent limitations of the HSV-*tk* suicide system is that only cells that progress through the S-phase of the cell cycle are killed by GCV administration. In essence, this means that only activated, rapidly proliferating T cells are eliminated efficiently, whereas cells that are in a resting, nonproliferation state are relatively resistant. In the clinical trial involving the infusion of HSV-*tk*–engineered lymphocytes, this resulted in the partial resistance of chronic GVHD to GCV treatment.[2,11] By contrast, Fas-induced apoptosis does not require S-phase entry.[27] The AP1903/Fas suicide system is, therefore, expected to function independently of the proliferative state of the cell. In addition, the transgene-derived Fas-fusion protein is expressed constitutively, and there is less than a 2-fold difference in expression levels between activated and resting T cells (data not shown). Therefore, AP1903-induced apoptosis should operate independently of activation-induced Fas-receptor up-regulation,[34] and it should be dependent only on the sequential activation of caspases, which are known to be constitutively expressed.[35-37]

The cell cycle dependence of the AP1903/Fas system was tested by taking advantage of the transient nature of the activation of in vitro–stimulated T lymphocytes. After the initial stimulation, cells proliferated rapidly and remained in a highly activated state for approximately 2 weeks, after which proliferation slowed down and they gradually returned to a less activated ("resting") state. To measure the proliferation/activation state of the Fas-engineered T cells, we examined 2 parameters: the expression of the IL-2 receptor α chain (CD25) and the incorporation of [^{3}H]thymidine.

As shown in Figure 6A, LV'VFas-transduced T lymphocytes expressing high levels of CD25 (top panel) were eliminated by AP1903 with 66% ± 7.5% (n = 10) efficiency. When cells were examined after CD25 expression returned to basal levels (Figure 6A, bottom panel), 63% ± 4.7% (n = 9) killing was observed after AP1903 treatment. Taken together, these results show that there is no statistically significant difference ($P = .17$) between the killing efficiencies of highly activated ($CD25^{hi}$) T cells and cells that have returned to a less activated state ($CD25^-$). We then analyzed cycling and noncycling LV'VFas-transduced T cells based on their incorporation of [^{3}H]thymidine. Again, there was no significant difference between rapidly cycling and noncycling cells with respect to their killing efficiencies (Figure 6B). These results demonstrate that the AP1903/Fas suicide system functions equally

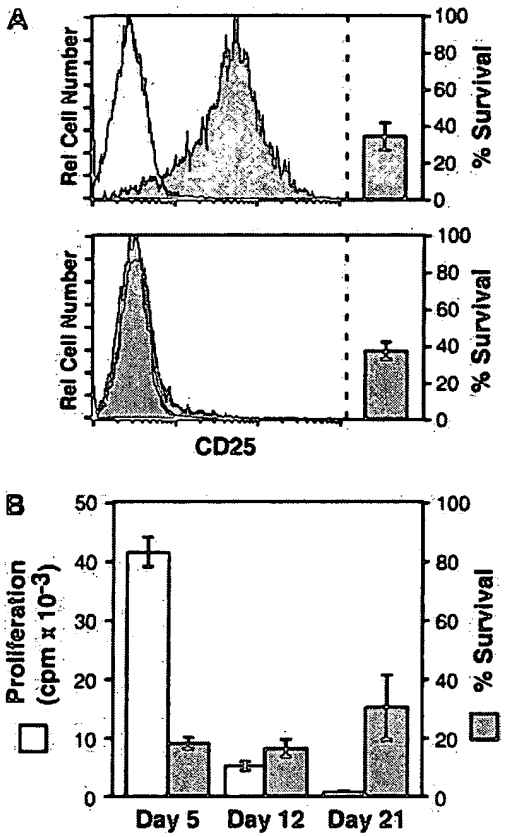

Figure 6. AP1903-induced killing is independent of the proliferation state of human T lymphocytes. (A) LV'VFas-transduced primary human T cells were stained for CD25 at different times after the initial stimulation, and the killing efficiency of $CD25^{hi}$ (top panel) and $CD25^{-}$ (bottom panel) cells was measured 24 hours after treatment with 10 nM AP1903. Representative CD25 staining profiles (filled histograms) with the isotype control (open histograms) are shown on the left side. Survival percentages of AP1903-treated cells are shown on the right side. (B) Five, 12, or 21 days after the initial stimulation LV'VFas-transduced primary human T cells were incubated with [^{3}H]thymidine in the absence of AP1903 to assess proliferation activity (open bars) or in the presence of 10 nM AP1903 to assess relative survival (closed bars). Values are the mean ± SD of 3 independent points.

well in rapidly cycling, highly activated T cells and in noncycling, resting T cells. This is in good agreement with the finding that stimulated primary human T cells expressing endogenous Fas could be killed with anti-Fas antibodies or FasL, regardless of their proliferation or activation state.[27]

Fas-transduced T lymphocytes are able to mount a specific antiallogeneic response

To determine whether Fas-transduced T lymphocytes are immune functional, and in particular whether they retain the ability to generate a specific antiallogeneic response, an MLR was performed. LV'VFas-transduced lymphocytes were stimulated with irradiated, allogeneic PBMCs, and specific cytotoxic activity was measured in a chromium release assay 10 days later. As shown in Figure 7A, LV'VFas-transduced T cells exhibited the same specific lytic activity against allogeneic target cells as untransduced T cells, suggesting that transduced cells are immune functional and that activation on encounter with a specific antigen does not trigger spontaneous apoptosis. Because donor T cells play a central therapeutic role during allo-BMT in providing a GVL effect, these properties are important for the eventual use of the AP1903/Fas system to combat GVHD.

To test whether the cytotoxic activity of Fas-engineered T cells can be inhibited by AP1903 treatment, cells from the primary MLR were restimulated with allogeneic PBMCs in the absence or presence of 10 nM AP1903. As expected, 60% to 80% of the Fas-engineered cells were killed by the drug in the secondary MLR (data not shown). Interestingly, cells surviving the drug treatment exhibited significant reductions in lytic activity relative to Fas-engineered cells restimulated in the absence of AP1903 (Figure 7B). This result suggests that in addition to the 60% to 80% reduction of cytotoxic activity associated with AP1903-induced cell death, the lytic activity against an allogeneic target cell of Fas-engineered T cells is significantly reduced after exposure to AP1903, an issue particularly relevant in the treatment of GVHD. This result is in good agreement with the previous observation that antiallogeneic lymphocytes challenged with the relevant target cell showed a reduction in proliferation of approximately 35% when they were preincubated with anti-Fas antibody.[38]

Discussion

In this report, we examined the feasibility of using the AP1903/Fas suicide strategy for the treatment of GVHD after allogeneic BMT by examining its performance in vitro in genetically engineered primary human T lymphocytes. We showed that a modified Fas cassette that incorporates a cell surface marker allows transduced cells to be purified to homogeneity without activating apoptosis. We demonstrated that a "codon-wobbled" version of F36V-FKBP is a crucial element to prevent the rearrangement of the Fas transgene, an important safety consideration in gene therapy applications using retroviral vectors. We showed that primary

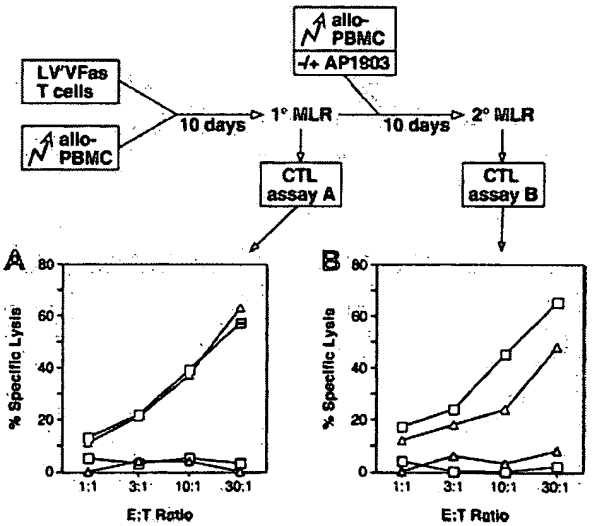

Figure 7. Immune potential of Fas-engineered T lymphocytes. A schematic illustration of the mixed lymphocyte reactions carried out is shown (see also "Materials and methods"). Untransduced or LV'VFas-transduced primary human T cells were incubated with allogeneic PBMC for 10 days (1° MLR). Cultures were divided in half, and a second MLR was performed by another addition of allo-PBMC and in the absence or presence of 10 nM AP1903 (2° MLR). The cytotoxic activity of equivalent numbers of cells from the 1° MLR or the 2° MLR was measured in a standard chromium release assay. (A) Cytotoxic T-lymphocyte (CTL) assay with cells from the 1° MLR: specific antiallo (Δ) and antiautologous (△) response of LV'VFas-engineered cells; and specific antiallo (▦) and antiautologous (□) response of untransduced cells. (B) CTL assay with cells from the 2° MLR: specific antiallo response of LV'VFas-engineered cells cultured in the absence (□) or the presence (Δ) of 10 nM AP1903; and antiautologous control response of LV'VFas-engineered (△) and untransduced cells (□).

human T lymphocytes can be engineered to express high levels of the Fas transgene and can then be eliminated by the AP1903/Fas system with high efficiency, potency, and specificity. Importantly, Fas-transduced lymphocytes retain their immune potential, a relevant issue for the GVL effect. The redesigned Fas cassette, therefore, fulfills the criteria we identified at the beginning of this study, required for clinical usefulness for combating GVHD.

Spencer et al[15,19] have found that expression of Fas in transiently transfected cells induces apoptosis in the absence of drug addition. Most likely this basal toxicity ("autotoxicity") is caused by the self-association of the death domain.[39] Consistent with these findings, we observed high autotoxicity with the LV'VFas construct in transiently transfected cell lines. However, when the LV'VFas construct was introduced in cells by retroviral infection, there was a complete lack of autotoxicity, both in cell lines and in primary human T lymphocytes. These findings suggest that autotoxicity is only observed when Fas is expressed at extremely high levels, as is the case in transiently transfected cells. This is in good agreement with the finding that Fas toxicity is significantly decreased when the amount of DNA transfected is reduced.[19]

Although this study was undertaken to investigate potential clinical use of the Fas T-cell suicide system, the conditional elimination of T is also useful as a research tool. Conditional ablation of subpopulations of T cells, for example in transgenic mouse models,[15,40] provides a means to probe their importance in specific aspects of the immune response. Our data suggest that the modified Fas construct with an integral cell surface marker could also be useful in these research applications.

Recent clinical experience with the HSV-*tk* suicide system has identified several significant limitations to its use in treating GVHD associated with allo-BMT.[11,41] Immune responses against infused HSV-*tk*–engineered T lymphocytes developed in several patients, resulting in the elimination of the cells and precluding the possibility of future infusions. In addition, chronic GVHD could only be partially controlled by GCV administration, a finding that was attributed to the cell cycle dependence of HSV-*tk* killing.[11] Finally, GCV administration for concurrent clinical conditions other than GVHD resulted in the undesired clearance of HSV-*tk* donor lymphocytes and, hence, in the loss of the GVL effect.

The AP1903/Fas system offers several potential advantages over the HSV-*tk*/GCV strategy. Unlike HSV-*tk*, all the functional protein components of AP1903/Fas are of human origin and are less likely to elicit immune responses. The only potential for immunogenicity in the construct are the point mutation in F36V-FKBP and 3 junction peptides. We demonstrate that with a single treatment of AP1903, 60% to 80% of Fas-engineered cells are eliminated. This is in comparison with 30% to 50% in vitro killing of HSV-*tk*–neo-engineered T cells[11] using the same construct (SFCMM-2) that was used successfully in clinical trials.[2] The most recent retroviral vectors carrying the wild-type HSV-*tk* suicide gene are more efficient in killing highly proliferating cells—a single administration of the prodrug results in more than 90% T cell deaths in vitro.[11] Although a single exposure of LV'VFas-transduced lymphocytes to AP1903 is not as efficient as GCV in killing transduced lymphocytes, LV'VFas-engineered T cells can be eliminated nearly completely by repeated exposure to the drug. Furthermore, killing of T cells expressing the Fas suicide gene is more rapid than that of cells expressing HSV-*tk* in vitro. Fas-engineered cells show signs of apoptosis within 1 hour of drug addition and killing is nearly complete after 24 hours, whereas cell death induced by HSV-*tk* occurs over a time course of several days. We provide evidence that AP1903/Fas-induced cell death, unlike HSV-*tk*,[11] occurs independently of the proliferation state of the cell. Thus, engineered T cells should be eliminated with high efficiency even in patients with chronic GVHD, in which GVHD effector cells likely are low-proliferating cells.[11] The drug used to trigger suicide in the Fas system is AP1903, a synthetic, small-molecule compound that was specifically designed to interact with the engineered F36V-FKBP but not with endogenous FKBP12.[16] In contrast, the HSV-*tk* system requires GCV, an antiviral drug with a low therapeutic index because of myelotoxicity[42] and moreover a drug broadly used for treatment of cytomegalovirus infection.[43]

Our experiments provide information that will be useful in defining the dosing regimen for use in GVHD applications. Specifically, delivery of short (1- to 2-hour) pulses of drug that are repeated at approximately 24- to 48-hour intervals should mimic the conditions found to induce efficient elimination of engineered T cells in vitro. The pharmacologic properties of AP1903 in healthy human volunteers were recently determined in a phase 1 trial that demonstrated the safety of the drug and established a direct relation between the plasma concentrations and the dose administered (J. D. Iuliucci, unpublished data). No clinically meaningful adverse effects were observed at plasma AP1903 concentrations up to 100-fold above the maximally effective in vitro concentration.

Based on our experiments showing safety, specificity, killing efficiency, and immune competence of LV'VFas-engineered lymphocytes and on the results of the phase 1 clinical trial of AP1903, we propose the AP1903/Fas system as a novel, safe, and efficacious suicide strategy to induce a controlled GVL effect in allogeneic marrow transplantation. Our data indicate that Fas-engineered donor lymphocytes should be as efficacious as HSV-*tk*–engineered donor lymphocyte infusions in controlling disease relapse but able to bypass the intrinsic limitations of the HSV-*tk*/GCV suicide system.

Acknowledgments

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Appendix 2

Molecular Medicine

GENE TRANSFER TO HEMATOPOIETIC CELLS

MALCOLM K. BRENNER, M.D., PH.D.

ALTHOUGH diseases of blood cells constitute a relatively small proportion of clinical disorders, many valuable studies of gene transfer have relied on hematopoietic stem cells or their progeny. What features of these cells explain their appeal in gene therapy? Perhaps most important is their hierarchical pattern of development (Fig. 1). Fewer than 1 in 100,000 bone marrow cells are pluripotent, self-renewing hematopoietic stem cells, but these rare cells yield progeny that multiply by many hundredfold and, in the process, follow a particular blood-cell lineage (monocytic, lymphocytic, granulocytic, megakaryocytic, or erythrocytic). These committed progenitors then multiply by a hundredfold or more to become the mature cellular components of the blood. Hence, in principle, genetic modification of small numbers of hematopoietic stem cells could provide a person with stable populations of genetically altered cells within each hematopoietic lineage. Hematopoietic stem cells are readily obtained from marrow or blood; they can be genetically manipulated outside the body and then returned to the patient after their functional status is verified. By contrast, in most other types of gene transfer the genetic material must be delivered in vivo to vast numbers of cells distributed over a wide anatomical area.

Another attractive feature of these cells for gene therapy is the wide variety of conditions that may be treated with genetically engineered hematopoietic stem cells. Most of these disorders affect hematopoietic cells (Table 1), but the presence of mesenchymal stem cells in bone marrow raises the possibility that gene-modified marrow-derived cells could be used to treat disorders of cartilage, bone, and muscle. And since the progeny of hematopoietic stem cells disperse throughout the body, they have potential usefulness as gene-delivery agents.

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To take full advantage of hematopoietic stem cells for gene transfer, the inserted gene must replicate at the same rate as the host cell, and all progeny of the host cell must receive equal doses of the gene. For all practical purposes, this means that the gene must integrate into the DNA of the host cell. At present, murine retroviruses are the only vectors that can unequivocally integrate DNA into cellular DNA. A human DNA parvovirus (adeno-associated virus) is a possible alternative, but its ability to integrate into the genome of hematopoietic stem cells in humans remains in question. Retroviral vectors have many limitations, among which is their inability to integrate into the DNA of nonproliferating cells. The potential benefits of retrovirus-mediated gene transfer are limited because few hematopoietic stem cells are in the cell cycle at any given time, a fact that reduces the efficiency of gene transfer.

There is little doubt that improvements in vector technology mentioned in earlier articles in this series will enhance the value of hematopoietic stem cells in gene transfer. In the meantime, various strategies are being applied to exploit existing capabilities. Despite its low efficiency, gene transfer into hematopoietic stem cells can mark cells for the purpose of tracking in vivo. This procedure has been applied in studies of marrow-ablative chemotherapy followed by rescue therapy with hematopoietic stem cells for the treatment of malignant diseases. It was not clear until recently whether relapses after such treatment were due to residual disease alone or to tumorigenic cells that contaminated the harvested stem cells, which of necessity are removed from the patient before the myeloablative treatment. By using retroviral vectors to label the harvested stem-cell population genetically before reinfusion, it was possible to show, in acute and chronic myeloid leukemias and neuroblastomas, that the rescuing population of hematopoietic stem cells was indeed contaminated with tumorigenic cells. Hence, gene transfer to hematopoietic stem cells revealed how reinfused autologous stem cells can contribute to the recurrence of disease, as well as allowing assessment of new techniques of purging autologous marrow or blood of neoplastic cells.

Studies of progenitor cells in blood that reconstitute the bone marrow after ablative chemotherapy are addressing questions about the biology and behavior of normal stem cells in vivo. For example, with the use of combinations of growth factors and stromal elements it may be possible to increase the rate of gene transfer by stimulating cell division in hematopoietic stem cells, which divide infrequently. However, this strategy has been only marginally effective.

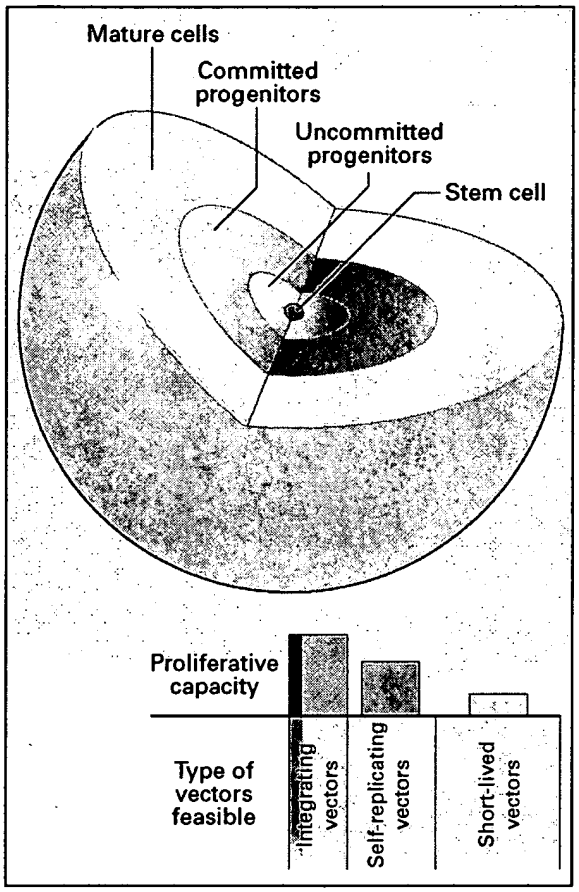

Figure 1. Hematopoietic Stem Cells and Their Progeny.

When stem cells divide, one daughter cell remains a stem cell and the other undergoes multiple divisions, forming uncommitted progenitor cells. These in turn divide and differentiate into committed progenitor cells. After further division, large numbers of mature cells are formed. Each component of the marrow has a different proliferative capacity. The variation in proliferative potential allows the selection of vectors with different characteristics at each developmental stage. At one extreme, the stem cell requires transduction (gene transfer) with a vector (such as a retrovirus) that integrates stably into its genome in order to affect a substantial number of blood cells. By contrast, post-mitotic or short-lived cells such as neutrophils may be transduced by vectors (such as an adenovirus) that do not integrate into host DNA or replicate themselves.

It also has the potential to cause hematopoietic stem cells to differentiate and lose their capacity to renew themselves, thereby negating the intent of the genetic manipulation. By using distinctive gene markers on populations of marrow cells exposed to different combinations of growth factors, it may be possible to track each population separately and so evaluate the consequences of particular treatments on the efficiency of gene transfer and the capacity of the treated hematopoietic stem cells to renew themselves.

Notwithstanding the limitations of available vectors, hematopoietic stem cells are still attractive targets for gene therapy. There are diseases in which the gene-corrected minority of stem cells could have a growth advantage over the uncorrected majority, such as severe combined immunodeficiency due to a lack of adenosine deaminase. The first gene-therapy protocols entailed transfer of the adenosine deaminase gene to cells from children with this congenital disease. This approach was based on the concept that only T lymphocytes derived from cells containing the transferred gene would survive and expand in vivo once the usual treatment with pegademase bovine was stopped. This idea remains to be formally tested, but the data obtained so far are encouraging. The same concept is applicable to Fanconi's anemia, in which correction of the underlying DNA-repair defect by gene transfer to hematopoietic stem cells might provide a selective growth advantage to a minority population of hematopoietic precursors.

An alternative approach to most genetic defects in hematopoietic cells is to introduce not only the gene of therapeutic interest but also a gene that gives the cell a selective growth advantage. Drug-resistance genes are an example of the latter type of gene. Preclinical data show that with this method a minority population of hematopoietic stem cells that express drug-resistance genes can be selectively amplified in the presence of the relevant cytotoxic drug. This approach is being tested in patients by transferring the multidrug-resistance gene to their hematopoietic stem cells, reinfusing the cells, and then beginning treatment with the cytotoxic agent paclitaxel. The intent of these studies is to devise methods of reducing the myelotoxic effects of cytotoxic drugs in order to allow intensification of the dose of these agents.

It may be possible to ignore the elusive hematopoietic stem cells altogether and focus instead on their lineage-committed progeny, which readily accept exogenous genes in a variety of vectors and which may survive long enough to be therapeutically useful. The limited capacity of these cells to multiply (Fig. 1) means that large numbers may need to be exposed to the vector to obtain a useful population of genetically modified cells. The problem of the relatively limited persistence of the transferred gene in mature cells can be overcome by repeated administration of vectors. The disorders that might be treated by this strategy range from clotting abnormalities to infections (Table 1). A good example of this application is gene transfer to T lymphocytes, which are readily induced to divide by cytokines, efficiently accept exogenous genes, and are relatively long lived. Moreover, by transferring a gene to antigen-specific T cells ex vivo, it is possible to induce proliferation of the cells in vivo by exposing the host to the appropriate antigen. Recently, genetically modified cytotoxic T cells were successfully used to treat disorders associated with the Epstein–Barr virus. Ex-

TABLE 1. EXAMPLES OF GENE THERAPY INVOLVING THE PROGENY OF HEMATOPOIETIC STEM CELLS.

LINEAGE	TYPE OF DISEASE	EXAMPLE
Lymphocyte	Immunodeficiency states	Severe combined immunodeficiency, acquired immunodeficiency syndrome
	Infections	Cytomegalovirus and Epstein–Barr virus diseases
	Tumors expressing specific or restricted antigens	Hodgkin's disease
	? Metabolic storage disorders	
	? Clotting disorders	
Erythrocyte	Hemoglobinopathies	Sickle cell disease
Monocyte	Metabolic storage disorders	Galactosialidosis
	Infectious diseases	Acquired immunodeficiency syndrome
	Functional defects	Chronic granulomatous disease
	? Clotting disorders	
Granulocyte	Functional defects	Chronic granulomatous disease
	Acute infections	
Megakaryocyte	Platelet disorders	

tensive efforts are now under way to develop T-cell–mediated gene therapies for patients with the acquired immunodeficiency syndrome.

It will be necessary to devise means by which relatively small numbers of genetically modified hematopoietic-cell progenitors can be made to compete for a niche with the much larger number of unmodified cells already entrenched in the host. Cotransfer of a gene that gives the cell a selective growth advantage may help to overcome this problem, but it remains likely that partial or complete ablation of preexisting marrow will be required. Such conditioning is used for the transplantation of allogeneic stem cells for disorders of hematopoietic cells, but there is a natural reluctance to apply these ablative regimens to the transfer of gene-modified autologous hematopoietic stem cells.

Gene regulation is also important in treatments involving genetically modified cells. Some gene products, such as adenosine deaminase, can benefit immunodeficient patients over a wide range of concentrations and can be constitutively produced without apparent adverse consequences; other gene products require much tighter regulation. For example, in an animal model of Bruton's agammaglobulinemia (Bruton tyrosine kinase knockout mice), constitutive expression of the missing Bruton tyrosine kinase gene in transplanted cells did not correct the immunoglobulin deficiency. The expression of this gene may need to be closely coordinated with developmental events in the cell. Hence, for many disorders involving hematopoietic stem cells, it may be necessary to construct vectors that carry not only the structural gene of interest, but also the considerable additional DNA required for gene regulation.

A further complication is that abnormal genes (or gene products) can inhibit the corrective gene. In sickle cell disease, continued production of the hemoglobin S β chains reduces the effectiveness of normal β chains produced by genetically modified cells. In addition to problems of gene regulation, there is concern that an immune response might neutralize the product of the transferred gene if it is normally absent in the host.

Many hematologic disorders may best be treated early in development, before the genetic abnormality causes irreversible changes. As the technology for the transplantation of fetal hematopoietic stem cells evolves, increased efforts to perform gene therapy in utero with these cells will be likely.

The logistic advantages inherent in the hematopoietic system will continue to make hematopoietic stem cells an appealing target for well-designed gene-transfer protocols that not only should be of clinical value but also should provide information of universal interest to those in the field.

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Appendix 3

Stimulating Cell Proliferation Through the Pharmacologic Activation of c-*kit*

By Liqing Jin, Haruhiko Asano, and C. Anthony Blau

Previous studies have shown that expression of a membrane targeted chimeric protein containing the erythropoietin receptor (EpoR) cytoplasmic domain fused to the FK506-binding peptide FKBP12 allowed Ba/F3 cells to be rescued from interleukin-3 (IL-3) deprivation using a dimeric form of FK506, called FK1012. In this report, a similar approach is applied to the c-*kit* receptor. Expression of a membrane targeted fusion protein containing the c-*kit* receptor linked to one or more copies of FKBP12 allowed Ba/F3 cells to be switched from IL-3 dependence to FK1012-dependence. Similar results were obtained using an alternative dimerizer of FKBP12 domains called AP1510. Pharmacologic dimerization of chimeric proteins containing only a single FKBP12 domain confirmed that receptor dimerization is sufficient for proliferative signaling. Interestingly, while the proliferative effects of both FK1012 and AP1510 were reversible, FK1012-driven proliferation persisted for several days after drug withdrawal. Furthermore, much higher concentrations of FK506 were required to inhibit FK1012-mediated proliferation than were required to inhibit AP1510-mediated proliferation. The persistence of FK1012's effect appeared to be specific to clones expressing c-*kit*–containing fusion proteins. These results suggest that pharmacologically-responsive fusion proteins containing c-*kit* may be useful for specifically and reversibly expanding genetically modified hematopoietic cell populations.

THE INEFFICIENCY of gene transfer into human hematopoietic stem cells poses a major obstacle for gene therapy of inherited and acquired blood cell disorders.[1,2] The development of therapeutic applications for stem cell gene transfer depends on markedly increasing the proportion of genetically corrected stem cells. One strategy for increasing the frequency of modified stem cells is to induce their preferential expansion through selection.[3] Providing the basis for selection are bicistronic vectors in which the nonselectable therapeutic gene is linked to a second gene encoding a selectable product. The frequency of genetically corrected cells can be increased by applying selection either ex vivo,[4,5] or if a clinically tolerable regimen were devised, selection could be applied in vivo. Current methods for in vivo selection involve the transfer of a gene conferring drug resistance such as multiple drug resistance-1 (MDR1) or dihydrofolate reductase (DHFR), followed by the subsequent administration of the corresponding cytotoxic drug in vivo.[6]

An alternative approach for selection would be to confer a direct proliferative advantage on the genetically modified cell population. The practical application of this strategy would require that the proliferative stimulus (1) be restricted to the genetically modified population and (2) be completely reversible. We have recently reported the use of a new method that appears to meet these requirements.[7] This approach is based on the principle that receptors for a variety of cytokines consist of single chains that are activated upon ligand-induced homodimerization.[8] Recently developed technology allows intracellular protein dimerization to be reversibly activated in response to a lipid soluble dimeric form of the drug FK506, called FK1012.[7,9-15] FK1012 is used as a pharmacologic mediator of dimerization to bring together two FK506 binding domains, taken from the endogenous protein FKBP12. Thus, fusion proteins consisting of a cytokine receptor signaling domain linked to an FKBP12 domain may be dimerized and thereby activated using FK1012.[9] This approach has been used to activate apoptosis through the Fas signaling pathway[15] and a related approach has been used to inducibly activate synthesis of a reporter gene in vivo.[16]

In recent studies,[7] we have shown that FK1012-induced aggregation of a fusion protein containing the intracellular portion of the erythropoietin receptor (EpoR) allowed Ba/F3 cells, which are normally dependent on interleukin-3 (IL-3), to proliferate in its absence. This approach meets two criteria that are essential for gene therapy applications: the proliferative stimulus is restricted to the genetically modified cell population, and it is reversible. In the context of the proper signaling molecule, a similar approach may be envisioned as a means for expanding genetically modified hematopoietic stem cells. Advantages of this system for in vivo applications are that FK1012 lacks the immunosuppressive properties of FK506[9] and that it can be administered at biologically effective doses.[15]

Toward the goal of using this approach for the expansion of genetically modified stem cells, we have tested the same strategy using a molecule, which upon activation has the reported capacity for causing stem cells to divide: c-*kit*.[17-19] In this report, we show that Ba/F3 cells expressing a membrane-targeted chimeric protein containing the intracellular domain of c-*kit* linked to one or more copies of FKBP12 are capable of FK1012-dependent proliferation in the absence of IL-3. These results suggest that pharmacologic dimerization of c-kit–containing fusion proteins may be useful for expanding genetically modified hematopoietic stem cells.

MATERIALS AND METHODS

Plasmid construction. F3, also designated pMF(PK)3E,[12] is a modified form of pMF3E.[9] FKBP12 has been modified to contain the mutations G89P and I90K. These mutations abrogate the ability of the FK506 complexes of this mutant FKBP to interact with calcineurin[20] and have a reduced propensity to interact with cellular proteins.[12] A 1296 or 1164 bp cytoplasmic domain of the murine *c-kit* was amplified by polymerase chain reaction (PCR) using *Pfu* polymerase, a plasmid

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containing the *c-kit* cDNA (a gift from Alan Bernstein, University of Toronto, Toronto, Canada) as a template and the following primers: 5′ CCC CTC GAG TAC AAA TAT TTG CAG AAA CC; 3′ (432 amino acids): CCC CTC GAG GGC ATC TTC GTG CAC; 3′ (388 amino acids): CCC CTC GAG CTT GGT GCT GTC CGA GAT. The PCR amplified fragments were digested using *Xho* I, gel purified, and inserted into an *Xho* I–digested and phophatased pBluescript vector. After sequence confirmation using the PRISM system (Applied Biosystems, Foster City, CA), the fragment was released from pBluescript by *Xho* I digestion and ligated into the phophatased *Sal* I digested plasmids F3 or F1 to generate the plasmids F3c-*kit*432, F3c-*kit*388 and F1c-*kit*432 (Fig 1). Plasmids were purified over two sequential cesium chloride gradients before transfection.

Electroporation. BaF3 cells were maintained in RPMI supplemented with glutamine, pyruvate, penicillin, streptomycin, 10% fetal calf serum, and 10% WEHI conditioned medium. Cells were split 1:2 on the day before transfection. Electroporations were performed as described previously.[7]

Western blotting. Cells were washed twice with phosphate-buffered saline (PBS), and approximately 1×10^6 cells were lysed in 100 μL 50 mmol/L Tris pH8.0, 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and placed on ice for 30 minutes. Western assays were performed using the HA.11 antibody (Berkeley Antibody Co, Berkeley CA), as previously described.[7]

Cell proliferation assay. Cell proliferation assays were evaluated by using MTT colorimetric assay (Sigma) as described previously.[7] FK1012 was a gift of David Spencer (Baylor University, Houston, TX), and AP1510 was a gift of Ariad Pharmaceuticals (Boston, MA).

RESULTS

Ba/F3 cells expressing F3c-kit432 proliferate in response to FK1012. Studies were performed to determine whether Ba/F3 cells expressing a c-*kit*/FKBP12 fusion protein can be induced to proliferate using FK1012. Initial studies were performed using the construct F3*c-kit*432 (Fig 1A). This construct was produced by inserting the cytoplasmic domain of the murine *c-kit* receptor into the *Sal* I site of the plasmid F3 (Fig 1A). F3c-*kit*432 encodes a chimeric protein containing a 14-amino acid myristylation-targeting domain from c-*Src*[21] to direct localization to the inner surface of the cell membrane, three copies of a 107-amino acid FKBP12 module[22] to bind the drug FK506, the entire 432 amino acid intracellular domain of the murine *c-kit* receptor, and a 9-amino acid influenza HA epitope tag[23] to allow detection of the recombinant protein by Western analysis.

Previous studies have shown that Ba/F3 cells expressing the full-length c-*kit* receptor are capable of proliferation with stem cell factor (SCF) in the absence of IL-3.[24] Experiments were performed to determine whether Ba/F3 cells expressing the F3*c-kit*432 fusion protein could, after withdrawal of IL-3, be rescued by FK1012. Ba/F3 cells were cotransfected with the F3*c-kit* 432 construct and a plasmid encoding neomycin phosphotransferase. G418-resistant clones were expanded and evaluated for expression of the chimeric protein by Western analysis (Fig 1B). Three Ba/F3 cell clones expressing high levels of the F3*c-kit*432 fusion protein were tested in cell proliferation assays. Proliferation failed to occur in the absence of IL-3–containing WEHI conditioned medium, and WEHI conditioned medium stimulated cell proliferation in a concentration-dependent manner (data not shown).

Proliferation assays were performed using the same clones in

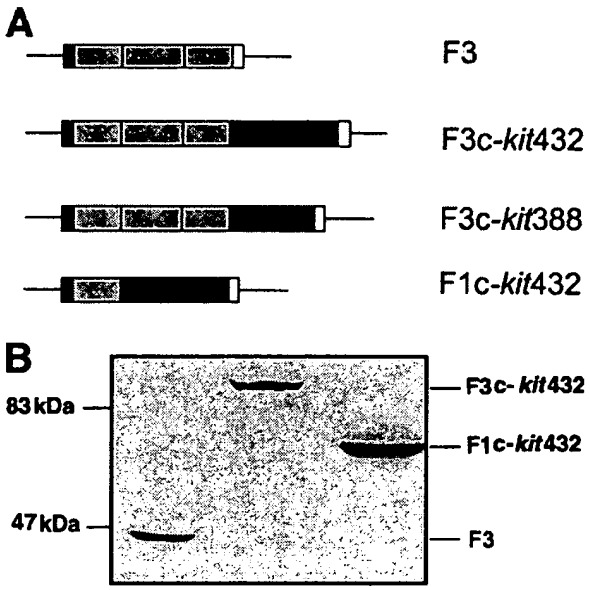

Fig 1. Plasmid constructs and Western blot. (A) Schematic representation of constructs. F3, previously designated pMFPK3E [12] is a modified version of pMF3E,[9] where the FKBP12 domain has been modified to contain the mutations G89P and I90K. Murine *c-kit* receptor sequences encode either the full-length 432-amino acid cytoplasmic domain, or the membrane proximal 388 amino acid cytoplasmic domain. (■), Myristylation peptide; (▤), FKBP12; (■), intracellular domain of *c-kit* receptor; (□), HA epitope tag. (B) Western blot of protein extracts from Ba/F3 cell clones expressing the F3 and F3c-*kit*432 constructs display protein bands of predicted sizes.

the absence of IL-3, but with the addition of either FK1012 or a new synthetic dimerizer of FKBP12 domains called AP1510. Concentrations of drug ranged from 10^{-2} to 10^3 nmol/L. As shown in Fig 2, both FK1012 and AP1510 exerted concentration-dependent proliferative effects in all three clones. To allow comparisons between the proliferative effects of FK1012, AP1510, and WEHI conditioned medium, results shown in Fig 2 indicate absorbance as a fraction of that obtained in parallel cell proliferation assays using 5% WEHI conditioned medium. For FK1012, cell proliferation was observed at as low as 1 nmol/L, peaking at concentrations of 100 nmol/L. Proliferation also occurred in response to AP1510, although approximately 10-fold higher concentrations of AP1510 were required to achieve a similar proliferative response. To show that the c-*kit* domain is required for mitogenic signaling, two control BaF3 clones expressing high levels of the construct F3, which lacks the *c-kit* domain (Fig 1), failed to proliferate in response to either FK1012 or AP1510 (data not shown).

Dimerization of the FKBP12/c-kit fusion protein is sufficient for proliferative signaling. The presence of three FKBP12 domains in F3c-*kit*432 could allow FK1012 or AP1510 to stimulate proliferation either through dimerization or through oligomerization of the chimeric molecule. To distinguish between these two possibilities for *c-kit* activation, a construct was produced in which the 432-amino acid *c-kit* receptor was linked to only a single copy of the FKBP12 domain (F1*c-kit*432) (Fig 1 A). Proliferation in response to FK1012 or AP1510 in cells

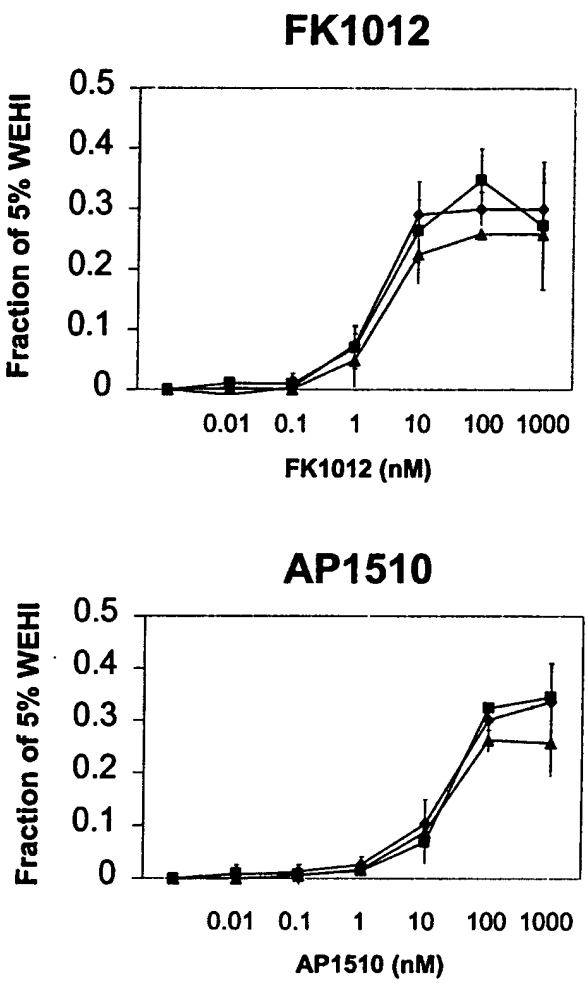

Fig 2. Pharmacologic activation of c-*kit* stimulates cell proliferation. MTT assays for three Ba/F3 clones expressing the construct F3c-*kit*432. Cells were tested in the absence of IL-3 and in the presence of either FK1012 (upper panel) or an alternative dimerizer of FKBP12 domains called AP1510 (lower panel) at concentrations ranging from 10^{-2} nmol/L to 10^{3} nmol/L. All Ba/F3 clones expressing F3c-*kit*432 showed a concentration-dependent proliferative response to both FK1012 and AP1510. Results are plotted as a fraction of OD570-630 nm values obtained using the same clone cultured in 5% WEHI conditioned medium. Data points indicate mean values of three separate experiments. Error bars indicate standard deviations. Note that in comparison to FK1012, approximately 10-fold higher concentrations of AP1510 are required to achieve equivalent proliferative effects.

expressing this fusion protein can be caused only by dimerization. As shown in Fig 3, five Ba/F3 cell clones expressing F1c-*kit*432 exhibited proliferative responses to both FK1012 and AP1510. In parallel assays, the level of cell proliferation observed in these clones appeared to be slightly reduced compared with clones expressing F3c-*kit*432. These results confirm that dimerization of c-*kit* is sufficient for mitogenic signaling.

FK506 efficiently competes FK506-mediated cell proliferation, but inefficiently competes FK1012-mediated cell proliferation. To test the hypothesis that FK1012 and AP1510 activate cell proliferation by bringing together FKBP12 domains of adjacent FKBP12/c-*kit* fusion proteins, competition assays were performed using the monomer FK506. Cells were grown in the presence of either FK1012 (100 nmol/L), AP1510 (100 nmol/L), or WEHI conditioned medium (1%), and a range of FK506 concentrations. Ba/F3 clones expressing either F3c-*kit*432 or F1c-*kit*432 were tested, and compared with a Ba/F3 clone expressing the construct F3EpoR236,[7] which contains the intracellular domain of the murine erythropoietin receptor inserted into the corresponding *Sal* I site of F3 (Fig 1A). In Ba/F3 cells expressing F3EpoR236, FK506 exerted a concentration-dependent inhibition of FK1012-mediated cell proliferation (Fig 4), identical to results reported previously.[7] Similar results were obtained in the presence of AP1510, although lower doses of FK506 were required for complete inhibition,

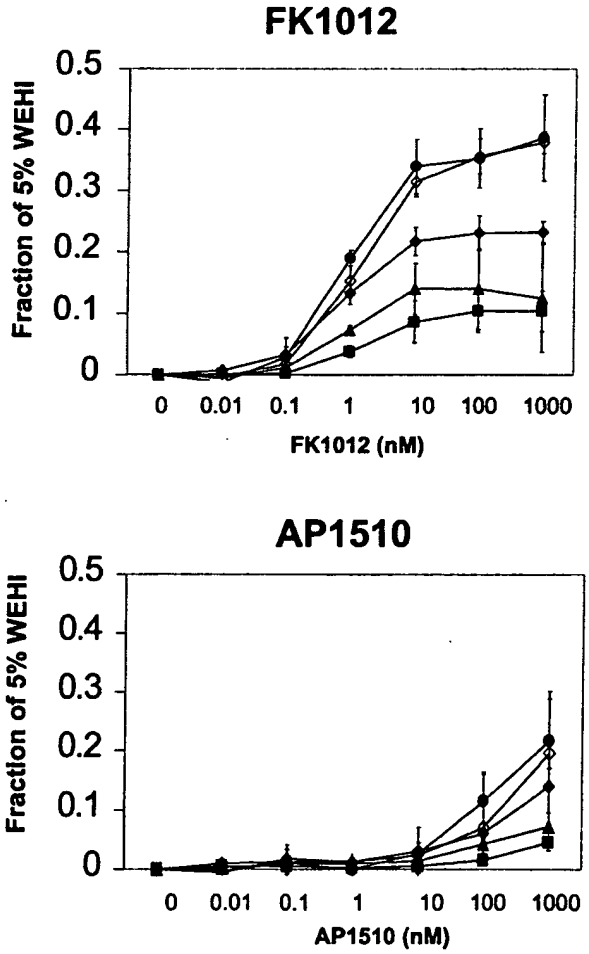

Fig 3. Dimerization of c-*kit* is sufficient for proliferative signaling. MTT assays were performed on five Ba/F3 clones expressing the construct F1c-*kit*432 in the absence of IL-3 and in the presence of increasing concentrations of either FK1012 (upper panel) or AP1510 (lower panel). Results are plotted as a fraction of the OD570-630 measured from the same cells cultured in 5% WEHI conditioned medium. Data points indicate mean values of three separate experiments. Error bars indicate standard deviations. Note that the level of cell proliferation observed is slightly reduced compared with Ba/F3 cell clones expressing the F3c-*kit*432 construct.

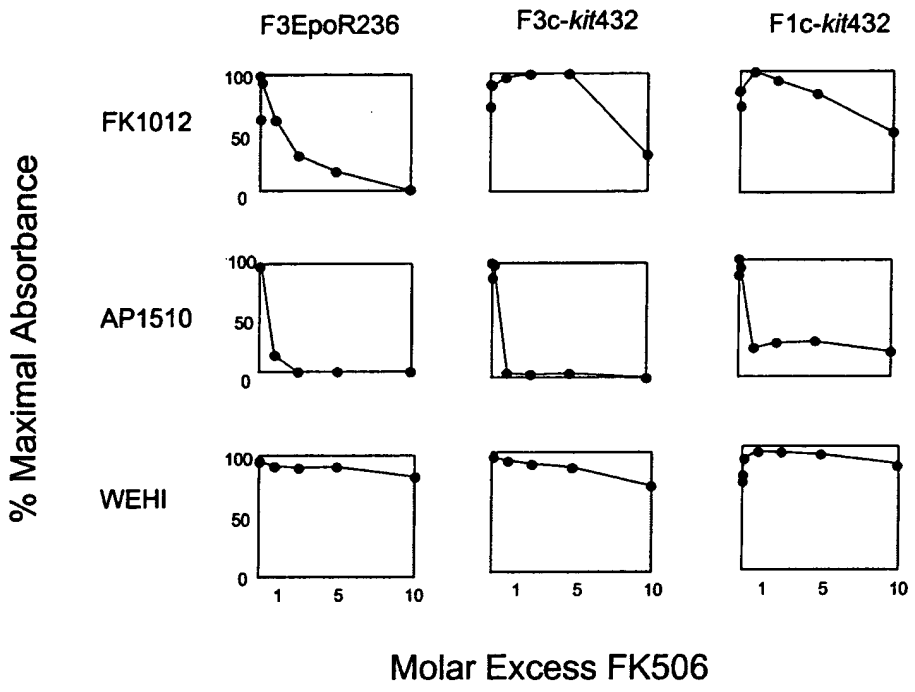

Fig 4. FK506 efficiently competes AP1510-mediated cell proliferation, but inefficiently competes FK1012-mediated cell proliferation. MTT assays were performed using clonal populations of Ba/F3 cells expressing F3EpoR236 (left column), F3c-*kit*432 (middle column), or F1c-*kit*432 (right column). Cells were grown in the presence of 100 nmol/L FK1012 (upper panels), 100 nmol/L AP1510 (middle panels), or 1% IL-3–containing WEHI conditioned medium (lower panels), plus FK506 at the indicated concentrations. Note that in the presence of WEHI conditioned medium, FK506 failed in all cases to inhibit cell proliferation (lower panels). In contrast, for each clone FK506 exerted a concentration-dependent inhibition of AP1510-mediated cell proliferation (middle panels). Similarly, in a Ba/F3 cell clone expressing F3EpoR236, FK506 produced a concentration-dependent inhibition of FK1012-dependent cell proliferation. In contrast, both Ba/F3 cell clones expressing fusion proteins containing c-*kit* (F3c-*kit*432 and F1c-*kit*432) were resistant to FK506-mediated inhibition of FK1012-dependent cell proliferation. Repeat experiments using the same clones and different clones expressing the same constructs yielded very similar results.

consistent with AP1510's approximately 20-fold lower binding affinity for FKBP12 relative to FK506.[24a] As expected, FK506 exerted no effect on IL-3–mediated proliferative signaling.

Surprisingly, evaluation of Ba/F3 cells expressing FKBP12/c-*kit* fusion proteins showed significantly different findings (Fig 4). In Ba/F3 cells expressing F3c-*kit*432, equimolar concentrations of FK506 failed to reduce FK1012-dependent cell proliferation, and a significant degree of proliferation persisted despite the presence of a 10-fold molar excess of competing monomer. At a 100-fold excess of FK506, complete inhibition of FK1012-dependent proliferation was finally observed. The same results were obtained irrespective of whether FK506 or FK1012 was added to the cells first. In contrast for AP1510, equimolar concentrations of FK506 resulted in a nearly complete inhibition of AP1510-mediated cell proliferation. These results suggest that while FK506 efficiently competes with AP1510 for access to FKBP12 sites, the monomer appears to be surprisingly less efficient in competing with its dimeric counterpart, FK1012. Similar results were obtained using F1c-*kit*432: FK506 efficiently inhibited AP1510-dependent cell proliferation, but inhibition of FK1012-mediated proliferation was highly inefficient (Fig 4). As expected, the doses of FK506 used had no significant effect on IL-3–mediated cell proliferation.

Persistence of FK1012-mediated cell proliferation after drug withdrawal. To confirm that the proliferative effects of FK1012 and AP1510 are reversible, cells initially cultured in the presence of either FK1012 or AP1510 were observed for cessation of cell growth after drug withdrawal. Ba/F3 cells expressing the F3c-*kit*432 fusion protein were plated in media without IL-3 supplemented with either FK1012 or AP1510 (both 100 nmol/L). As shown in Fig 5, cell proliferation was observed in response to either compound, while in the absence of either drug or IL-3, cells died over a period of 2 to 3 days. Similar to results obtained in the MTT assays, FK1012 appeared to be a slightly more potent stimulator of cell proliferation than was AP1510 at the concentrations examined. After 3 days of culture, cells grown initially in FK1012 or in AP1510 were washed extensively and then cultured either in the presence or absence of added drug. Cells grown initially in AP1510 died within 24 to 48 hours after drug withdrawal. In contrast, cells grown initially in FK1012 continued to slowly proliferate for up to 7 days after FK1012 was withdrawn. Thereafter, continued deprivation of FK1012 eventually resulted in cell death (Fig 5). Similar results were obtained with Ba/F3 cell clones expressing the F1c-*kit*432 fusion protein (data not shown). Taken together with results from the competition assays, these findings suggest that FK1012 binding to the FKBP12/c-*kit* fusion protein may persist for several days after drug withdrawal.

Deletion of the carboxy terminal end of the c-kit receptor reduces responsiveness to FK1012 and AP1510. Deletion of the carboxy-terminus of a related receptor c-*fms*, results in an increased receptor sensitivity to ligand-dependent activa-

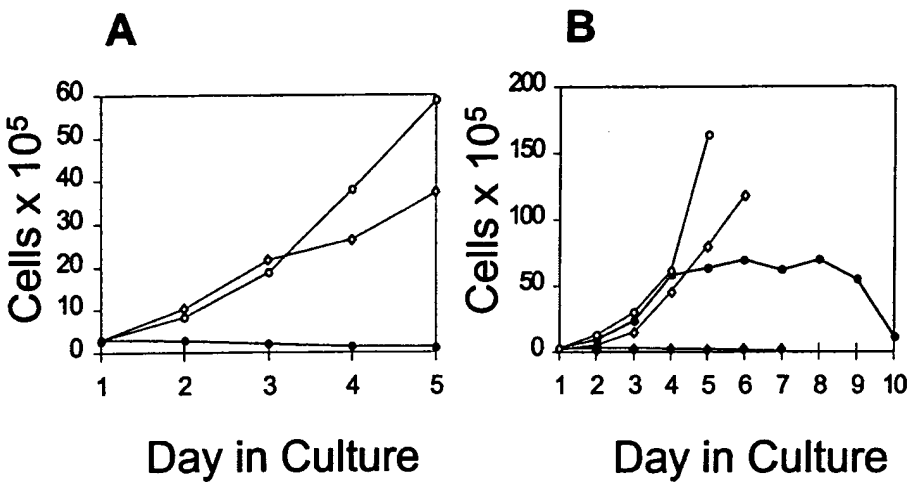

Fig 5. Persistence of FK1012-mediated cell proliferation after drug withdrawal. (A) A clonal population of Ba/F3 cells expressing F3c-*kit*432 proliferates in IL-3–deficient medium supplemented with either FK1012 (○) or AP1510 (◇) (both 100 nmol/L). In the absence of either drug or IL-3 (●), cells died over a period of 2 to 3 days. (B) The same cells grown initially in FK1012 or in AP1510 were washed extensively and then cultured either in the presence or absence of added drug. In the continued presence of FK1012 (○) or AP1510 (◇), continued cell proliferation was observed. After withdrawal of AP1510 (◆), cell death occurred over 1 to 2 days. In contrast, cells grown initially in FK1012 continued to proliferate for up to 1 week after FK1012 was withdrawn (●). Very similar findings were observed in a repeat experiment.

tion.[25,26] To determine whether negative growth regulatory domains reside in the carboxy terminal tail of c-*kit*, the construct F3*c-kit*388 was produced, in which the carboxy terminal 44 amino acids of c-*kit* were deleted (Fig 1A). Five BaF3 clones expressing the F3*c-kit*388 were generated. As shown in Fig 6, both FK1012 and AP1510 exerted concentration-dependent proliferative effects in the absence of IL-3. However, in parallel comparisons with BaF3 clones expressing F3*c-kit*432, truncation of the *c-kit* receptor appeared to significantly decrease responsiveness to FK1012 and AP1510. These findings are not attributable to differences in the level of receptor expression (data not shown).

DISCUSSION

Fundamental to the success of stem cell gene therapy is the development of methods for increasing the frequency of genetically corrected stem cells. This task may in theory be accomplished through selection. Selection can be applied either ex vivo, or if a clinically tolerable approach were devised, repeated cycles of selection could potentially be applied in vivo. Current approaches for in vivo selection involve the transfer of a drug resistance gene into a small population of hematopoietic cells. Selective pressure is applied through in vivo administration of the appropriate cytotoxic drug. Success requires that the cytotoxic drug exert a proportionally greater toxic effect on the population of unmodified marrow cells relative to their transduced counterparts. Recent studies have underscored a major problem in using drug resistance genes for in vivo selection: early hematopoietic cells tolerate very high dosages of the chemotherapy drugs to which these genes confer resistance.[27,28] Thus, chemotherapy provides little or no selective advantage to clonogenic progenitors bearing the drug resistance gene, as these early hematopoietic cells are normally highly drug-resistant.[27-30]

An alternative method for accomplishing selection would be to confer a direct proliferative advantage on the genetically modified cell population. The clinical applicability of this approach would mandate that the proliferative advantage be reversible. The use of pharmacologic inducers of dimerization[7-13] may provide a method for achieving this goal. Using fusion proteins containing the erythropoietin receptor, we have recently reported the development of a "proliferation switch."[7] In Ba/F3 cells expressing the FKBP12/EpoR chimeric protein, the addition of FK1012 switches cell proliferation to the "on" position, and withdrawal of FK1012 switches proliferation to the "off" position. Toward the goal of testing whether a similar approach might be used for the expansion of genetically modified hematopoietic stem cells, we tested this strategy using c-*kit*.

Mutations at the white spotting (*W*) and steel (*Sl*) loci provide the most compelling evidence for a biologic role of c-*kit* and its ligand, respectively, in stem cell regulation.[31,32] Normal marrow cells introduced into W/W^v mice can repopulate the entire hematopoietic system without conditioning, indicating a severe defect in the repopulating ability of stem cells in these animals.[33] Further evidence comes from the severe pancytopenia that results from administration of an antibody directed against c-*kit* in mice.[34] C-*kit* ligand (SCF) administration in vivo stimulates the redistribution and expansion of progenitors.[35-37] Furthermore, SCF administration produces a threefold expansion in the total number of transplantable stem cells in mice when given alone[17] and a 10-fold expansion when given in combination with granulocyte colony-stimulating factor (G-CSF) in splenectomized mice.[19]

In this report, we show that Ba/F3 cells expressing FKBP12/c-*kit* fusion proteins can proliferate in response to either FK1012 or a new synthetic FKBP12 dimerizer, called AP1510. After IL-3 withdrawal, Ba/F3 cells expressing the F3c-*kit*432 fusion protein could be rescued and maintained in either FK1012 or AP1510. Approximately 10-fold higher concentra-

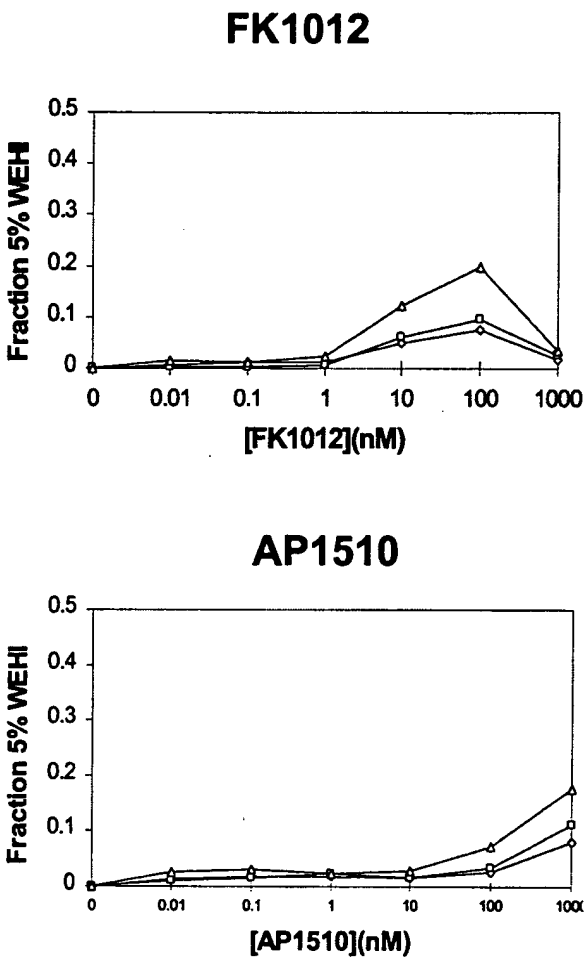

Fig 6. Deletion of the carboxy-terminal 44 amino acids of c-kit reduces proliferation in response to FK1012 and AP1510. MTT assays were performed on three Ba/F3 clones expressing F3c-*kit*388, in which the carboxy terminal 44 amino acids of the c-*kit* receptor were deleted. Each clone responded to both FK1012 (upper panel) and AP1510 (lower panel), however, the level of proliferation was less than in clones expressing F3c-*kit*432 (Fig 2). Results are plotted as a fraction of the OD570-630 value measured from the same cells cultured in 5% WEHI conditioned medium. A second experiment yielded very similar results.

tions of AP1510 were required to achieve a level of proliferation similar to that observed with FK1012, possibly due to AP1510's 20-fold lower binding affinity for FKBP12 domains.[24a]

Several findings in this report differ from our previous observations using the erythropoietin receptor. First, in the context of the erythropoietin receptor, a fusion protein containing three FKBP12 domains was significantly more efficient in stimulating FK1012-mediated cell proliferation than was a fusion protein containing only a single FKBP12 domain.[7] In the case of c-*kit*, the difference in the level of proliferation observed in clones expressing fusion proteins with a single FKBP12 domain appeared to be only slightly reduced compared with clones with fusion proteins containing three FKBP12 domains. These results suggest that dimers may be nearly as efficient as oligomers in the stimulation of c-*kit*-mediated proliferative signaling. Retaining only a single FKBP12 domain has potential advantages for the development of retroviral vectors with regard both to the reduction in insert size and to the avoidance of repeated sequences that may promote recombination.

Unexpectedly, fusion proteins containing c-*kit* require extremely high concentrations of FK506 to inhibit FK1012's proliferative effect. Equimolar concentrations of FK506 had no discernible effect on FK1012-dependent cell proliferation, and even a 10-fold molar excess of competing monomer failed to completely inhibit FK1012's effect. In contrast, FK506 efficiently inhibited AP1510-driven cell proliferation. Furthermore, FK506 efficiently competed with FK1012 in parallel experiments using a clone expressing F3EpoR236. These observations suggest that c-*kit* may in some way stabilize the interaction between the FKBP12 moiety of the fusion protein and FK1012. The hypothesis that FKBP12/c-*kit* fusion proteins bind FK1012 in a highly stable manner is supported by our observation that cell proliferation persists for up to 1 week after FK1012 withdrawal (Fig 5). In this regard, it is noteworthy that after prolonged maintenance in IL-3–containing cultures (12 to 16 weeks), some Ba/F3 cell clones expressing FKBP12/c-*kit* acquire the capacity for factor independent cell growth, but remain FK1012 responsive (data not shown). The significance of these findings awaits testing in transduced primary murine bone marrow cells.

In view of the short in vivo half lives of both FK1012 and AP1510 (Spencer et al[15] and Amara et al[24a]), drug concentrations attainable in vivo are likely to be low. Thus, signaling molecules that can be activated at very low drug concentrations may be advantageous. Several mutations in the cytoplasmic domain of c-*kit* have the capacity to trigger constitutive receptor activation and neoplastic transformation.[38,39] Sequence differences between the feline v-*kit* oncogene and feline c-*kit* include the replacement of the carboxy-terminal 44 amino acids in c-*kit* by five unrelated amino acids in v-*kit*. Other reports indicate that negative regulatory domains may reside within the carboxy terminal end of another class III receptor tyrosine kinase, *c-fms*.[25,26] In our studies, deletion of 44 amino acids from the carboxy terminal end of c-*kit* failed to augment responsiveness to either FK1012 or AP1510 and appear, in fact, to have resulted in an impairment of receptor function. The lack of an increase in receptor sensitivity is in agreement with a previous report and suggest that remaining sequence differences between v-*kit* and c-*kit* require evaluation.[40]

An eventual goal of our studies is to identify signaling domains that stimulate stem cells to divide, but not to differentiate. Pharmacologic activation of wild-type c-*kit*, which is also a major regulator of mast cell differentiation,[41-44] is unlikely to fulfill this goal. Urgently needed are approaches for the identification and, if possible, the elimination of domains necessary for maturational signaling.

Although our studies show that FK1012 or AP1510 can rescue a factor-dependent cell line, demonstrating a proliferative advantage in genetically modified primary cells may be more difficult, particularly for in vivo applications. In Ba/F3 cells, withdrawal of IL-3 provides a strong selective pressure that is not reproducible in vivo. Whether pharmacologic inducers of dimerization can provide a proliferative stimulus in

transduced primary cells beyond that provided by a physiologic milieu of cytokines remains to be determined.

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2003-present	Member, Fred Hutchinson/University of Washington Cancer Consortium
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Haskill Schiff Award, Duke University Department of Medicine, 1989 (awarded to one resident annually for excellence in clinical medicine).

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Rotary Club Exchange Student to Ravensburg, Germany, July 1977 – June 1978.

PRESENTATIONS AT NATIONAL AND INTERNATIONAL MEETINGS

Invited Speaker, "

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Invited Speaker, Thirteenth Conference on Hemoglobin Switching, Oxford, UK September 25-29, 2002.

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Invited Speaker, Eastlick Symposium, Washington State University, March 2001.

Invited Speaker, Hemoglobin Switching Conference, Orcas Island, 2000.

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Invited Speaker, Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, October 8, 1999.

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Science in Medicine Lecturer, University of Washington, November 1998.

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Invited Speaker, Japanese Biochemical Society's Conference on Stem Cell Commitment and Expansion, Tsukuba, Japan, July 8-10, 1998.

Invited Speaker, Second Conference on Stem Cell Gene Therapy, Orcas Island, WA, June 1998.

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Conference on In Utero Transplantation and Stem Cell Gene Therapy, 1996

Hemoglobin Switching Conference, Orcas Island, 1996

First Conference on Stem Cell Gene Therapy, 1995

Hemoglobin Switching Conference, Orcas Island, 1994

American Society of Hematology, 1994

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Member, Special Study Section for Tissue Engineering, March 24, 2001.

External Referee for the Medical Research Council's Molecular Hematology Unit, Oxford, England, April 2001.

External Referee for Swiss National Science Foundation,April 2001.

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RESEARCH SUPPORT

A. Active Support

R01 DK52997 (Blau) 8/10/97 – 7/31/05 15%
NIH/NIDDK $179,000 (annual direct support)
A Proliferative Switch for Genetically Modified Cells
Specific Aim 1 will humanize the mpl vector. Specific aim 2 will test the humanized vector in normal mice and in a mouse model of mpl deficiency. Specific aim 3 will test the humanized vector in a canine model. Specific aim 4 will test the humanized vector in hemopoietic cells taken from patients with mpl deficiency.
No overlap.

R01 DK57525-02 (Blau) 9/1/99-8/31/03 (no cost extension) 15%
NIH/NIDDK $250,000 (annual direct support)
Mixed Chimerism in the Hemoglobinopathies
Specific Aim 1 tests whether CIDs can expand genetically modified stem and progenitor cells in vivo. Specific Aim 2 develops a mouse model of mixed chimerism in beta thalassemia, and seeks to determine the level of normal donor stem cell engraftment needed to reverse the thalassemic phenotype. Specific Aim 3 tests whether CID-mediated in vivo expansion of normal donor stem cells can correct the thalassemic phenotype of mice with mixed chimerism. Specific Aim 4 evaluates whether FL can sensitize stem ells to 5-FU. In Specific Aim 5, the FL/5-FU combination is added to an immunosuppressive condition regimen for studies in allogeneic models of mixed chimerism. In Specific Aim 6, findings from the previous specific aims are combined to test CID-mediated in vivo expansion of normal donor stem cells in an allogeneic model of mixed chimerism. Specific Aim 1 overlaps with Specific Aim 1 of P01HL53750.

R01 DK61844-01 (Blau) 9/1/01-8/31/05 20%
NIH/NIDDK $225,000 (annual direct support)
Hemopoietic Stem Cell Plasticity
Here we propose to characterize the developmental potential of human hemopoietic cells, and to develop a method that will allow for the pharmacologically controlled *in situ* expansion of cells that have transited from hemopoietic to non-hemopoietic tissues, using liver as a model. Our approach involves expressing a protein that induces cell growth in the presence of a chemical dimerizing agent. In Specific Aim 1 we will test the developmental potential of human hemopoietic cells. We will study autopsy tissues taken from female recipients of male bone marrow cells to determine whether male cells contribute to various non-hemopoietic tissues. In Specific Aim 2 we will test whether genetically modified hemopoietic cells retain hepatocyte potential. Bone marrow from male mice will be transplanted into female recipients who will then be analyzed for hepatocytes arising from the

male donor. In Specific Aim 3 we will construct and test vectors for expanding marrow derived hepatocytes. In Specific Aim 4 we will use chemical dimerizing agents to expand marrow-derived hepatocytes, *in vivo*.

P01 HL53750-02 (Stamatoyannopoulos) 9/01/00-8/31/04 10%
NIH/NHLBI $169,360 (annual direct support)
Subproject 4: Stem Cell Expansion Using Chemical Inducers of Dimerization (Blau).
The specific aims of the project are: 1) to test whether CID-mediated activation of mpl allows for the in vivo selection of transduced murine stem and progenitor cells; 2) to identify and eliminate mpl maturational signaling domains to produce a derivative that is capable of proliferative signaling but incapable of maturational signaling; 3) to test whether CID-mediated activation of mpl allows for the selection and expansion of transduced human CD34+ cells in vitro and in vivo; 4) to test in vivo selection in a large animal model using CIDs; 5) to test vectors that contain both a CID-selectable gene and a γ globin gene for studies of selection in normal mice and in a mouse model of sickle cell anemia. No overlap.

P01 DK55820-01-A1 (Stamatoyannopoulos) 5/01/00-4/30/05 10%
NIH/NIDDK $104, 519 (annual direct support)
Subproject 2: Receptor Specificity in the Proliferation and Differentiation of Hemopoietic Stem Cells (Blau)
Studies described in this proposal will identify and characterize the features of mpl signaling that are permissive for stem cell expansion. Experiments will be performed to determine whether mpl's ability to stimulate stem cell expansion is shared by the GCSF receptor. Finally, transgenic mice will be used to quantitate the magnitude and kinetics of stem cell expansion occurring in response to mpl and GCSF receptor signaling. Studies will be performed to determine whether transient activation of the mpl and GCSF receptor can influence the developmental fate of stem cells. No overlap.

U01 HL66947-01 (Stamatoyannopoulos) 9/28/00-8/31/05 5%
NIH/NHLBI $181,953 (annual direct support)
The UW/FHCRC Program of Excellence in Gene Therapy, Core A: Clinical Core (Blau)
The goal of this program is to combine the resources of two institutions, the University of Washington and the Fred Hutchinson Cancer Research Center, to advance gene therapy in two areas of interest to NHLBI- stem cell gene therapy of hematological disorders and gene therapy of two common inherited lung diseases. No overlap.

M01 RR00037-41 (Ramsey) 12/1/02-3/31/08 20%
NIH/NCRR $6,433,420 (annual direct support for GCRC)
General Clinical Research Center: Associate Program Director for Gene and Cell Therapy (Blau)
The goal of the General Clinical Research Center is to provide research infrastructure to the faculty members at the University of Washington, Children's Hospital & Regional Medical Center, Fred Hutchinson Cancer Research Center, the Seattle VA and Harborview Medical Center. No overlap.

1P20 GM69983-01 (Blau) 9/30/03-8/31/06 15%
NIH/NIGMS $520,338 (annual direct support)
UW/FHCRC Exploratory Center for Human ES Cell Research (Blau) $500,000/yr (requested)
This application proposes to build an infrastructure for human embryonic stem cell research that will serve both the University of Washington and the Fred Hutchinson Cancer Research Center.

Fundamental to this effort will be the establishment of a Human Embryonic Stem Core Laboratory, which will provide a centralized facility for the culture, maintenance, manipulation, and differentiation of human ES cells, and will serve as a resource for support and training of the local research community. Three Pilot Projects will complement the Core Laboratory to establish a foundation for human ES cell research in Seattle

PATENTS

USPTO Application No. 09/582,916 filed October 2, 2000 Entitled: "Methods of Controlling Cell Differentiation and Growth using a Fusion Protein and a Drug" UWOTL 2211-2733-3471PT Inventors: C. Anthony Blau and David Spencer.

LICENSE

Washington State Medical License number 0026522; 1989 - present

PROFESSIONAL ORGANIZATIONS

American Society of Clinical Investigation
American Society of Hematology
American Society of Gene Therapy

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5. Palena A, Blau A, Stamatoyannopoulos G, and Anagnou NP: Molecular characterization of a novel ($\delta\beta$)-thalassemia deletion with increased expression of fetal hemoglobin in a family of Eastern European origin. **Blood** 83:3738-3745, 1994.

6. Stamatoyannopoulos G, Blau CA, Nakamoto B, Josephson B, Li Q, Liakopoulou E, Pace B, Papayannopoulou Th, Brusilow S, and Dover G: Fetal hemoglobin induction by acetate, a product of butyrate catabolism. **Blood** 84:3198-3204, 1994.

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31. Masuko M, Weinreich M, Chien S, and Blau CA: Longterm Monitoring of Mpl-Based In Vivo Selection Using Chemical Inducers of Dimerization *In preparation.*

32. Zhao S, Weinreich M, Blau CA: In vivo selection using a JAK2-based cell growth switch. *submitted.*

Book Chapters

1. Blau CA and Stamatoyannopoulos G: Evolving therapies in globin chain disorders. In: Lechner K and Gadner H (eds): Haematology Trends '93, Schattauer Verlag, 1993.

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4. Blau CA: Current status of stem cell therapy and prospects for gene therapy. In: Rodgers G (ed): Sickle Cell Disease and Thalassemia. Baillieres Clinical Haematology, 11(1): 257-275, 1998.

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6. Blau CA: Selection of primary hemopoietic cells using chemical inducers of dimerization. In: Ikada Y, Shimizu Y (ed): Tissue Engineering for Therapeutic Use. Elsevier Science, 4:113-120, 2000.

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2. Blau CA: Induction of fetal hemoglobin with erythropoietin (letter). **Nephron** 65:336, 1993.

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23. Siritanaratkul N, Heimfeld S, Skarpidi E and Blau CA: Temporary interruption of hydroxyurea as a method for achieving progenitor cell mobilization I patients with sickle cell anemia. Blood 92:160a, 1998.

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32. Emery DW, Tubb J, Otto KG, Stamatoyannopoulos G, Blau CA: Use of a pharmacologically regulated cell growth switch to circumvent chromosomal position effects on globin gene vectors. Molecular Therapy 3: S150, 2001.

AUGMENTED CV

Carl Anthony Blau

TEACHING

1. I headed the red cell section of the hematology course for second year medical students and taught one of the small groups.

2. Research trainees:
   Tobias Neff, MD: 1/95-12/96, 6/00–4/02.
   Liqing Jin, MD - Hematologist, Beijing: 5/96 – 10/99
   Noppadol Siritanaratkul, MD - Hematologist, Thailand; 1/97 – 12/98.
   Robert E. Richard, MD, Ph.D. – Assistant Professor, Hematology, UW. 7/97- 1/03.
   Hui Zeng, MD – Hematologist, Beijing: 3/98 – 2/00.
   Shengming Zhao MD – Hematologist, Beijing, 5/99 – 12/02.
   Masayoshi Masuko MD – Hematologist, Japan, 10/99 – 4/02.
   Irina Kirillova, MD, Ph.D. Research in liver diseases. 8/00 – 7/03.
   Horst von Recum, PhD–Embryonic stem cells, 10/01-present.
   Wenjin Guo PhD-alternative dimerization systems. 2/02-present.
   Kenji Ihara, MD, Assistant Professor, Kyushu University, Japan, 7/02-present
   Yasuo Nagasawa, Ph.D., Lead Researcher, Sankyo, Tokyo, Japan, 10/02 - present
   Lazar Dimitrov, Graduate Student, Genome Sciences 7/03 – present
   Angelo DeClaro, MD, Hematology Fellow 9/03 – present
3. I organize the monthly meeting of the Seattle Gene Therapy Club, and the biweekly meeting of Stem Cell Club.

RESEARCH IN PROGRESS

*In vivo selection using a cell growth switch (*Jin et al., **Nature Genetics,** 26:64-6, 2000; Neff et al., **Blood**, 100:2026-2031, 2002, Zhao et al., in preparation).
Selection allows rare cells with a desired phenotype to emerge from a background of unwanted cells. Selection of cells within a living organism, termed *in vivo* selection, has the potential to overcome many of the current obstacles to gene therapy. Strategies for achieving *in vivo* selection have relied on genes that confer resistance to subsequently administered cytotoxic drugs, however these approaches entail toxicity to the organism as a whole. We have developed an alternative system for *in vivo* selection that uses a "cell growth switch," allowing a minor population of genetically modified cells to be directly, inducibly and specifically amplified, thereby averting the risks associated with cytotoxic drugs. This system provides a general platform for conditionally expanding genetically modified cell populations *in vivo* that may have widespread applications in gene and cell therapy. We have shown that this approach works in mouse and canine models. Studies in the primate model are ongoing. More recently, we have used the same approach to develop a JAK2-based cell growth switch.

Defining the signals that specify stem cell self-renewal (Zeng et al., **Blood** 98:328-334, 2001; Zhao et al., **EMBO J**, 21:2159-2167, 2002).
Defining signals that can support the self-renewal of multipotential hemopoietic progenitor cells (MHPCs) is pertinent to understanding leukemogenesis and may be relevant to developing stem cell-based therapies. We have defined a set of signals, JAK2 plus either c-kit or flt-3, which together can support extensive MHPC self-renewal. Phenotypically and functionally distinct populations of MHPCs were obtained, depending on which receptor tyrosine kinase, c-kit or flt-3, was activated.

Self-renewal was abrogated in the absence of STAT5a/b, and in the presence of inhibitors targeting either the mitogen activated protein kinase (MAPK) or phosphotidylinositol 3' kinase (PI-3K) pathways. These findings suggest that a simple two-component signal can drive MHPC self-renewal.

Selective expansion of genetically modified primary human hemopoietic cells using chemical inducers of dimerization (Richard et al., **Blood** 95:430-6, 2000, Richard et al., **Stem Cells 21:71-78, 2003**).

We've extended our studies using the dimerizer system to the selective expansion of transduced CD34+ cells of human cord blood origin. Transduced cells expanded an average of 186 fold in the presence of dimerizer. The responsive cell type was primarily erythroid. In more recent studies we have extended these observations to CD34+ cells from adult marrow. Furthermore, we have found that the cell lineages capable of responding to dimerizer can be modulated through the addition of growth factors. This work was described in an article published in the April 21, 2000 issue of the *Wall Street Journal* (page B6).

The first clinical gene therapy trial using chemical inducers of dimerization. In conjunction with Stan Riddell and our collaborators at Ariad Pharmaceuticals we are in the process of preparing to perform a clinical gene therapy trial in relapsed leukemia. Donor lymphocyte-mediated anti-tumor effects represent the single most important therapeutic benefit of allogeneic bone marrow transplantation (BMT). Paradoxically, donor lymphocyte-mediated Graft versus Host Disease (GVHD) represents the single greatest toxicity of allogeneic BMT. Over the past decade a large body of research has focused on harnessing the therapeutic potential of donor lymphocyte infusions while avoiding the development of life threatening GVHD. This proposal describes a pilot study that will test a new system that allows the survival of infused donor lymphocytes to come under pharmacological control. Donor lymphocytes are equipped with a suicide gene. In the past, HSV thymidine kinase has been used for this purpose, however the immunogenic nature of the HSV-TK protein will severely impede the use of this gene in future gene therapy trials. In order to reduce the likelihood of immunogenicity, it would be highly desirable to employ a suicide gene encoding a protein that is completely human in origin. Our collaborators at ARIAD Pharmaceuticals have developed such a system based on the human cell surface receptor Fas, which naturally signals apoptosis (programmed cell death) in T lymphocytes. Fas signaling is normally initiated by clustering of the receptor by its ligand, leading to a cascade of cytotoxic events. In the ARIAD system, clustering of an artificial Fas receptor (introduced by gene transfer) and consequent cell death is brought under the control of a small molecule drug Binding of this "dimerizer" clusters the chimeric Fas receptors and initiates the natural apoptotic cascade. All the protein components of this system are human in origin, therefore the peptide sequences at the fusion sites and a point mutation in FKBP12 represent the only potentially immunogenic sequences. 30 patients receiving donor lymphocyte infusions for relapse of hematological malignancy will be enrolled. We plan to begin enrollment into this trial in September 2002.

Other Applications of the Dimerizer System

We have embarked on a series of collaborative studies to determine the utility of using chemical inducers of dimerization to stimulate expansion of genetically modified liver cells (with Andre Lieber), pancreatic beta cells (with Ake Lernmark and Andre Lieber), and muscle cells (with Charles Murry). If successful, these approaches might prove useful for treating liver diseases, diabetes, and other disorders.

Preliminary Studies for Gene Therapy in Sickle Cell Disease and β Thalassemia

In order to perform gene therapy for these disorders, we will need to procure stem cells. The most widely used method for obtaining stem cells is to promote their mobilization into the peripheral blood using the cytokine GCSF so that they can then be collected by leukapheresis. We have previously shown that GCSF can produce life-threatening complications in patients with sickle cell disease (*Lancet*, 1998).

Our experience with GCSF strongly suggests that alternative means will need to be found for the procurement of stem cells in patients with sickle cell disease. A protocol to study the use of hydroxyurea for the mobilization of progenitors in patients with sickle cell anemia has been approved by the GCRC's Scientific Advisory Committee and the UW Human Subjects Committee, and has enrolled three patients. In collaboration with Bob Richard and Erica Jonlin, we are also examining the safety of stem cell collection in patients with sickle cell anemia. This work is part of the Program for Excellence in Gene Therapy.

Development of a Clinical Gene Therapy Program at the University of Washington
As Associate Program Director for Gene and Cell Therapy at the GCRC I have taken part in the effort to establish a world class gene therapy program at UW. Over the last 2 years I worked with Dr. Oliver Press to establish his gene therapy program on the CRC. I am also Director of the Clinical Core for the Program for Excellence in Gene Therapy, and am building the infrastructure for clinical gene therapy trials at UW.

PATIENT CARE ACTIVITIES

1. I attend on the Hematology Consultation Service for one to two months per year, and on the stem cell transplant service or general oncology another month per year.

2. I maintain a relatively busy Hematology Clinic one half day per week, with emphasis on patients with sickle cell anemia and β thalassemia.